STRUCTURAL AND FUNCTIONAL ANALYSIS OF THE ENDOMETRIAL GENE ENCODING INSULIN-LIKE GROWTH FACTOR-BINDING PROTEIN-2

By

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To my parents with deepest appreciation.

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The Insulin-like Growth Factors (IGFs) play important growth regulatory roles in the uterus. The functions of the IGFs are modulated by their receptors (type-I and type-II) and at least six distinct IGF binding proteins (IGFBPs), which are expressed in a tissue- and developmental-specific manner. In porcine uterus of pregnancy, insulin-like growth factor-binding protein-2 (IGFBP-2) is expressed in endometrial cells and accumulates at the feto-maternal interface. However, the function(s) of this protein and the molecular mechanisms of IGFBP-2 gene regulation in the uterus remain unknown. To address these questions, the chromosomal organization of the porcine IGFBP-2 gene was first characterized. This gene spans ~29 kb and is comprised of four exons and three introns. Exons 1, 2, 3 and 4 are ~527, 227, 141 and 528 bp, respectively. Introns 1, 2 and 3 are ~24 kb, 746 bp and 2.6 kb, respectively. The mRNA transcript is ~1.6 kb in size, and encodes a 316 amino acid

precursor. The TATA-less GC rich promoter was characterized and shown to have two clusters of transcriptional start sites (-109 and -78 relative to the transcriptional initiation codon). Subsequently, 1.4 kb of 5' flanking region was analyzed by deletion mutagenesis and transient transfection in ECC-I and JEG-3 cell lines, and epithelial and stromal cells isolated from pregnant pig endometrium. Results identified a 110 bp region from nucleotides-874 to -765 that has transcriptional stimulatory activity, whereas regions from nucleotides -1397 to -875 and -764 to -306 appeared to have cell-type dependent inhibitory activities. Within the 110 bp region, two consensus sequences TCAGGG and CCCTGA were identified by gel retardation assay to bind the same nuclear protein designated A2 DNA binding protein with molecular weight of 33 kDa as estimated by Southwestern blot analysis. Estrogen stimulated endometrial IGFBP-2 gene expression *in vivo* in the ovariectomized pig model and *in vitro* in primary cultures of endometrial cells. Ligand blot analysis showed that the binding capacity of IGF-II receptors on uterine cell membranes was correlated with IGFBP-2 gene expression in these same cells. IGF-II, Phorbol 12-Myristate 13-Acetate (PMA) and cyclic AMP increased in IGFBP-2 mRNA abundance in endometrial cells. In contrast, TGF-β1 and IGF-I did not alter IGFBP-2 mRNA levels. *In vitro* DNA methylation at the promoter region did not alter the rate of transcriptional initiation of this gene in a HeLa cell-free transcription system. Moreover, no differential DNA methylation between a high IGFBP-2 expressing tissue (endometrium) and low IGFBP-2 expressing tissues (myometrium and placenta) was found for several CpG doublets within the 5' end of the gene. Lastly, recombinant porcine IGFBP-2 protein (rpIGFBP-2) was expressed and purified from E.coli cells. This rpIGFBP-2 exhibited biological activity as monitored by IGF-binding. In conclusion, distal promoter region of the porcine IGFBP-2 gene is important for this gene regulation. IGF-II may be

responsible for pregnancy stage-specific IGFBP-2 gene expression, whereas estrogen may be responsible for estrous cycle-related IGFBP-2 gene expression. Availability of functional rpIGFBP-2 may provide a means to elucidate the endometrial functions of IGFBP-2.

CHAPTER 1
INTRODUCTION

The Insulin-like Growth Factor (IGF) system probably is one of the more complicated functional control systems required for orchestration of a number of biological processes, particularly with respect to growth and development. Insulin-like Growth Factor-I (IGF-I) and Insulin-like Growth Factor-II (IGF-II), as major participants of this system, are mutifunctional polypeptides with structural similarity to proinsulin. Although some of the functions of IGF remain unclear, studies have demonstrated that IGFs can affect cell proliferation, differentiation, cell cycle progression, function and death. These two peptides are synthesized in a variety of fetal and adult mammalian tissues, and function not only in an endocrine but also autocrine and paracrine fashion. These actions of IGF are mediated through two different cell membrane receptors with different affinities for ligand. IGF-I receptor (also called the type 1 receptor) is a heterotetramer with structural and functional similarity to the insulin receptor, while IGF-II receptor (also called the type 2 receptor) has a single transmembrane domain, and is identical to the cation-independent mannose 6-phosphate receptor. The most complicated aspect of the IGF system is the involvement of the IGF binding proteins (IGFBPs), a family of related proteins with at least six members (IGFBPs-1 to -6). In the extracellular fluid, IGFs usually are bound to one or more of these binding proteins with high affinity. This binding modifies the action of IGFs in several

different ways. IGFBPs can act as carriers in the circulation and thereby prolong IGF biological half-life. Because they bind to IGFs with higher affinity than do IGF receptors, IGFBPs can localize IGFs to target cells. Interestingly, IGFBPs appear to have IGF-independent functions as well. The exact biological roles of each IGFBPs are still not determined. Functions of the IGFBPs appear to be altered by post-transcriptional modifications, such as phosphorylation, glycosylation and proteolysis. Therefore, there is no single model to describe all the features of this system. In different tissues and physiological conditions, the IGF system can exhibit different functions by virtue of the unique combination of participating proteins.

The uterus plays an important role in supporting embryo growth and development. The uterine endometrium is an epithelial-mesenchymal tissue lining the lumen, which undergoes morphological and biochemical changes during the estrous cycle and throughout pregnancy. In pregnant animals, the uterine endometrium, in response to signals from the conceptus (embryo and its associated membranes), provides a suitable environment for implantation and subsequent development. This feto-maternal interaction involves both endocrine controls, such as utero-ovarian interactions, and local autocrine and paracrine controls involving proteins, such as growth factors and cytokines. The IGF system is involved in controlling uterine functions. For example, during periimplantation, the porcine endometrium synthesizes peak amounts of IGF-I. This IGF-I stimulates embryonic aromatase gene expression, which in turn enhances estrogen production from the embryo. Estrogen, as a signal for maternal recognition of pregnancy, acts on the endometrium to block the $PGF_{2\alpha}$ luteolytic signal production or secretion. Therefore, the corpus luteum is maintained

as is production of progesterone, which is required for pregnancy. IGFs, IGF receptors and IGFBPs are differentially expressed in uterine and fetal tissues, and undoubtedly have important roles in uterine cell proliferation and differentiation.

IGFBP-2 exhibits a particularly interesting expression pattern in porcine uterus. This gene is expressed in the endometrium, but not in the myometrium and placenta of pregnant pigs. In endometrium, this gene is expressed at low levels during early pregnancy and is highly expressed during mid- and late-pregnancy. This uterine-synthesized IGFBP accumulates on the surface of endometrial epithelial cells. These observations raise two important questions: 1). What is the molecular mechanism(s) for this tissue- and developmental stage-specific pattern of gene expression? 2). What is the function of this protein at the feto-maternal interface? The objectives of this study were to gain a better understanding of the molecular basis of uterine IGFBP-2 gene expression, and to provide information concerning the functional aspect of endometrial IGFBP-2. The specific hypotheses examined were as follows: 1). The distal regulatory region of the endometrial IGFBP-2 gene is important for its transcriptional expression. 2). Use of this gene as a model may lead to identification of novel transcription factor(s) and *cis*-element(s) underlying endometrial-associated gene expression.

CHAPTER 2
LITERATURE REVIEW

Insulin-like-Growth Factors and Their Binding Protein Family

Insulin-like Growth Factors

Background. The Insulin-like Growth Factors (IGFs) were discovered on the basis of three separate biological activities. Sulfation factor activity (SFA) was defined by Salmon and Daughaday (1957), who observed that the growth-promoting action of Growth Hormone was mediated by a substance in serum that stimulated the uptake of sulfate by costal cartilage in explant cultures. The term nonsuppressible insulin-like activity (NSILA) was coined by Froesch et al. (1966) based on the observation that the insulin-like action of serum on muscle and adipose tissue could not be abolished by inclusion of anti-insulin antibody. BRL-3A rat hepatoma cell conditioned medium had mitogenic activity, and this was termed multiplication-stimulating activity (MSA) by Dulak and Temin (1973). In 1972, the term somatomedin was used to denote uncharacterized factors that mediated the action of Growth Hormone in the stimulation of somatic growth and that displayed insulin-like activity (Daughaday et al., 1972).

In 1978, Rinderknecht and Humbel purified two distinct somatomedin peptides from human serum. Base on the sequence and structure homologies with human proinsulin, IGF-I and IGF-II were used to designate these peptides. In 1987, the term IGF-I was officially

recommended to refer to the somatomedins A and C, which were found to be identical to IGF-I (Klapper et al., 1983; Enberg et al., 1984) and IGF-II was recommended to replace the designation MSA (Daughaday et al. 1987).

Peptides. IGF-I and IGF-II are single-chain polypeptides with three intrachain disulfide bonds consisting of 70 (7.6 kDa) and 67 (7.5 kDa) amino acids, respectively (Rinderknecht and Humbel, 1978a, b).These two peptides can be subdivided into B, C, A and D domains beginning from the N-terminus. The structure of the A and B domains are homologous to the A and B chains of insulin. Similar to proinsulin, the C domain separates the A and B domains. Unlike for proinsulin, however, a D domain is found at the C-terminus of IGFs.

Gene structures. The genes encoding IGF-I and IGF-II are single-copy (Tricoli et al., 1984; Brissenden et al., 1984). IGF-I genes have been characterized in human (Rotwein et al., 1986), rat (Shimatsu and Rotwein, 1987), Sheep (Dickson et al., 1991), and chicken (Kajimoto and Rotwein, 1991). The IGF-I gene consists of six exons (reviewed by Sara and Hall, 1990; LeRoith and Roberts, 1993). Exons 1 and 2 encode alternative 5'-untranslated regions and ATG translation initiation codons for the precursor proteins. Exons 3 and 4 encode the B, C, A, and D domains of the peptide. Exons 5 and 6 encode the extended E peptide, which is post-translationally processed, and contain 3'-untranslated sequences. The chicken IGF-I gene does not have an exon 2 encoding a alternative 5'-untranslated region nor an exon 5 encoding the E peptide. Multiple transcription start sites in exons 1 and 2, alternative splicing in exons 1 and 5, and multiple polyadenylation sites in exon 6 produce

a collection of IGF-I mRNAs containing divergent 5'- and 3'-untranslated regions as well as the region encoding the E peptide.

The structure of the IGF-II gene has been elucidated for the human (Bell et al., 1985; de Pagter-Holthuizen et al., 1988), rat (Frunzio et al., 1986; Chiariotti et al., 1988) and mouse (Rotwein and Hall., 1990). Similar to the IGF-I gene, the structure of the IGF-II gene consisting of 9 exons is quite complicated (reviewed by LeRoith and Roberts, 1993). Exons 1 through 6 encode divergent 5'-untranslated regions. There are 4 different promoters (P1-4) in front of exons 1, 4, 5, and 6, respectively. Exons 7 and 8 encode the IGF-II peptide domains B, C, A, and D. Exon 9 encodes part of the E peptide and also contains the 3'-untranslated region. The combination of differential promoter usage, alternative splicing and multiple polyadenylation sites results in 6 different IGF-II mRNA species with the sizes of 6.0, 5.3, 5.0, 4.8, 2.2 or 1.8 kb. All of these transcripts, except for the exon 9-derived 1.8 kb-species, encode the same IGF-II precursor. Promoter 1 is active in hepatic cells after birth. Promoters 2, 3 and 4 are predominantly utilized in embryonic cells (Sara and Hall, 1990). Interestingly, the IGF-II gene is closely linked to the insulin gene, which is located only 1.4 kb from exon 1 of the IGF-II gene (Bell et al., 1985).

Actions of the IGFs. The biological functions of IGF-I and -II have been reviewed intensively (Sara and Hall, 1990; Lowe 1991; Giudice, 1992; Cohick and Clemmons, 1993; and Jones and Clemmons, 1995). The *in vitro* biological actions of IGF-I exhibited the following aspects: 1. Effects on cell cycle progression. IGFs stimulate DNA synthesis and cell replication causing cells to traverse successive phases of the cell cycle, in particular from G_0 to G_1. 2. Effects on cell proliferation. A wide variety of cell types demonstrate a

mitogenic response to exogenous IGF-I. 3. Inhibition of cell death. 4. Stimulation of cell differentiation and function. For example, IGF-I and -II stimulate hormone synthesis and secretion by ovarian granulosa and theca cells (Giudice, 1992).

The *in vivo* studies of the effects of IGF administration have clearly demonstrated the insulin-like action of IGFs (Tomas et al., 1992, reviewed by Jones and Clemmons, 1995). Mice with null mutations of the IGF-I and IGF-II genes had birth weights that were 60% of normal mice while relative body proportions were normal (DeChiara et al., 1990; Liu et al., 1993a). Mice with a null mutation for both IGF-I and IGF-II had 30% of normal body weight, and all such mice died within minutes after birth due to respiratory failure (Baker et al., 1993). Overexpression of human IGF-I in transgenic mice increased body weight by 30% over control mice (Mathews et al., 1988). Overall, in transgenic mice models, IGFs exhibited prenatal growth-promoting activities.

Insulin-Like Growth Factor Receptors

There are two known receptors that specifically interact with the IGFs. The IGF-I receptor (also called the type 1 IGF receptor) shares a high degree of sequence similarity with the insulin receptor. The IGF-II receptor (also called the type 2 IGF receptor) is identical to the cation-independent mannose 6-phosphate receptor.

IGF-I receptor. Intensive studies of the IGF-I receptor were reviewed recently by LeRoith et al. (1995). The IGF-I receptor contains two α-subunits and two β-subunits that form a heterotetrameric structure similar to that of the insulin receptor (Massague and Czech, 1982). The entire α subunit protrudes extracellularly and contains a cysteine-rich domain, which is required for ligand binding. The β subunit contains the transmembrane spanning

domain and a cytoplasmic region with the highly conserved tyrosine kinase catalytic domain as well as several tyrosine residues which can be autophosphorylated. The α and β subunits are linked by a single disulfide bond to form an αβ-half-receptor. Subsequently, two of these αβ-half-receptors become linked by a disulfide bond between the α subunits to form a α2β2 heterotetrameric structure. The α and β subunits are generated from a single precursor encoded by a chromosomal gene with 21 exons (Abbott et al., 1992). The peptide cleavage site for generation of the subunits is the basic tetrapeptide Arg-Lys-Arg-Arg, which is encoded by exon 11.

Because of the similarity between IGF-I and insulin receptors, the αβ-half-receptor of these receptors can form hybrid insulin/IGF-I receptors. This hybrid receptor has been isolated from solubilized placental membranes (Soos et al., 1989; 1993) and cultured cells (Moxham et al., 1989). The affinities of receptor hybrids were lower for insulin than for IGF-I (Frattali and Pessin, 1993; Soos et al., 1993).

Signal transduction from the IGF-I receptor appears to be similar to that from the insulin receptor. IGF-I binding to the α subunits induces IGF-I receptor autophosphorylation of three tyrosine residues in the kinase domain of the β-subunit, and this results in activation of the intrinsic tyrosine kinase activity of the IGF-I receptor (Gronborg et al., 1993; Kato et al., 1993; 1994). The activated IGF-I receptor is able to phosphorylate other tyrosine-containing substrates. Interestingly, IGF-I receptor shares a common motif "PL-X4-NPXYXSXSD" (so-called insulin/IL-4R motif) with insulin and interleukin-4(IL-4) receptors within the intracellular domain (Wang et al., 1993a, b; White and Kahn, 1994; Keegan et al., 1994). After ligand binding, this motif becomes (auto-)phosphorylated and consequently

binds IRS-1 and other proteins to transduce the signal to the cell interior. IRS-1, the major substrate of the activated insulin receptor, is a predominant substrate of the IGF-I receptor as well (LeRoith et al., 1988; Myers et al., 1993a, b). When the gene encoding IRS-1 was inactivated, the IRS(-/-) mice were both insulin and IGF-I resistant, as manifested by marked intrauterine growth retardation (~50% of normal body weight) and a blunted response to the hypoglycemic action of injected IGF-I and insulin (Kahn et al., 1995). The residual biological effects in those mice were associated with the appearance of an alternative substrate of the receptor, IRS-2 which also appears to be 4PS, a substrate of the IL-4 receptor. IRS-1, which has multiple tyrosines in YMXM motifs, is considered to be a "docking" protein, that can bind SH2 domain-containing proteins. IGF-I receptor can potentially influence multiple intracellular signaling pathways through phosphorylation of IRS-1 (Sun et al., 1993), and its subsequent binding to proteins such as PI-3 kinase, MAP kinase, Grb-2, Nck (also an adaptor protein) and Syp (a phosphotyrosine phosphatase). These substrates and multiple signaling pathways, which also involve many other components, may be important for the diversity of biological functions described for IGF ligands and receptors in different tissues. In different cell types and different developmental stages, the presence of these substrates and other components for each pathway may differ, thereby resulting in different cell responses elicited.

IGF-II receptor. Un like the IGF-I and insulin receptors, the IGF-II/Man-6-P receptor is a monomeric glycoprotein. Human IGF-II receptor has a predicted molecular mass of 270 kDa including a long extracellular domain comprised of 15 Cys-rich repeats (~150 amino acids for each repeat), a small transmembrane region (23 amino acids) and a short

intracellular domain (164 amino acids) (Morgan et al., 1987). This receptor has distinct binding sites for IGF-II and Man-6-P or Man-6-P-containing glycoproteins, and can bind IGF-II and Man-6-P simultaneously (Braulke et al., 1988). Cell surface IGF-II receptors have a high affinity for IGF-II, a low affinity for IGF-I and no affinity for insulin (Nissley et al., 1991). There is a 240 kDa truncated form of IGF-II receptor (extracellular domain) that is found in the blood stream (MacDonald et al., 1989; Bobek et al., 1991). The function of this truncated soluble receptor form is, however, unknown. The gene encoding IGF-II receptor (*Igf2r*) is subject to maternal imprinting, whereas the gene encoding IGF-II is paternally imprinted (Barlow et al., 1991; DeChiara et al., 1991; Filson et al., 1993). When the IGF-II receptor gene was inactivated, the mice (*Igf2r-/-*) developed edema, and 100% of the fetuses died *in utero* at day 15 of gestation. However, when both the IGF-II receptor and IGF-II genes were knocked out, ~60% of the double mutant mice survived to birth (Filson et al., 1993). These authors speculated that excess IGF-II may have toxic effects *in utero*. In the case of mutation of the IGF-II receptor, the toxic effect of IGF-II may predominate, whereas in the case of the double mutation this toxic effect is no longer evident. However, this model remains speculative.

In contrast to the IGF-I receptor, the signal transduction pathway(s) of the IGF-II receptor is less clear. Unlike the IGF-I receptor, the short intracellular domain of the IGF-II receptor lacks tyrosine kinase catalytic activity (Czech, 1989; Ullrich and Schlessinger, 1990). Studies done by Nishimoto and colleagues have contributed to some understanding of the signaling by this receptor. In 1987, these workers found that IGF-II stimulated Ca^{++} influx in BALB/c 3T3 fibroblasts (Nishimoto et al., 1987). Subsequently, they reported that

the IGF-II receptor directly interacts with a G protein family, G_{i2} (Nishimoto et al., 1989; Murayama et al., 1990; Okamoto et al., 1990b). Subsequent analysis (Okamoto et al., 1990a) revealed a Gi couplone, which later was found to be a consensus region in the intracellular domain of most (if not all) G-protein-coupled receptors (Nishmoto et al., 1993; Nishimoto 1993).This couplone with a length of between 10 and 26 residues requires 2 basic residues on the N-terminal end and the sequence B-B-X-B or B-B-X-X-B at the C-terminus (where B is a basic residue and X, a nonbasic residue). This couplone has G-protein activating function. Moreover, it was found that these G-protein signals are interchangeable, by creating a chimeric IGF-II receptor (βIII-2/IGF-II receptor) containing a βIII-2 couplone sequence (Takahashi et al., 1993).

IGF Binding proteins (IGFBPs)

IGFs in the circulation are always associated with their cognate binding proteins. This phenomenon was discovered some 30 years ago, with the observation that the M_r of NSILA became reduced after treatment with 5 M acetic acid (Burgi et al., 1966). IGF binding proteins that bound [^{125}I]IGF with high affinity were first reported by Zapf et al. (1975). However, in only the most recent decade were the genes of at least six distinct IGFBPs cloned and intensive studies done on these proteins functions, gene expression pattern and genetic regulation (reviewed by Drop et al., 1992; Rechler and Brown, 1992; Jones and Clemmons, 1995).

Characteristics of the IGFBPs. All six IGFBPs are secretory proteins that generally do not accumulate in intracellular site(s). Therefore it can be difficult to measure the intracellular levels of these proteins. The different IGFBPs are clearly distinct molecules that

do share regions of strong similarity (Drop et al., 1992). Specifically, the hydrophobic cysteine-rich N-terminal and C-terminal regions are conserved. The alignment of these 18 cysteines is strongly conserved across the six IGFBPs with the exception of IGFBP-6 (lacks 2 cysteines in the human protein) and IGFBP-4 (contains 2 extra cysteines in the human and rat proteins). The middle one-third region, where no cysteines are present, is the most divergent among IGFBPs. IGFBPs-3, -4, -5 and -6 are glycosylated proteins. IGFBP-3 and -4 are N-glycosylated, whereas IGFBPs-5 and -6 are O-glycosylated. IGFBPs-I, and -2 contain an RGD sequence near the carboxyl terminus, which is involved in binding to the extracellular matrix protein receptors or integrins (Jones et al., 1993; Delhanty and Han, 1993b).

Functions of IGFBPs. It has been demonstrated that IGFBPs have multiple functions (recently reviewed by Jones and Clemmons, 1995) including the transport of IGFs in the circulation, mediation of IGF transport from the vascular compartment, localization of IGFs to specific cell types, modulation of IGF binding to receptors, and subsequent biological actions. IGFBP-1 is a 25 kDa protein found in high concentrations in amniotic fluid, and is also secreted by hepatocytes. IGFBP-2 has a significantly higher affinity for IGF-II than for IGF-I, and is the major fetal IGF binding protein. IGFBP-3 is the main carrier of IGF-I and -II in postnatal and adult serum. At least 95% of the total content of IGF-I and IGF-II in serum is bound to IGFBP-3. In plasma, IGFBP-3 and IGFs can form a 150 kDa complex. IGFBP-4 is a 24 kDa protein that was identified in serum and seminal plasma. IGFBP-5 and IGFBP-6 have recently been cloned, and these proteins exhibit a higher affinity for IGF-II than for IGF-I (Shimasaki, et al, 1991a, b).

The functions of IGFBPs appear to be altered by posttranslational modifications, such as glycosylation, phosphorylation and proteolysis. In human pregnancy serum, IGFBP-3 proteolytic activity is responsible for the disappearance of intact IGFBP-3 from serum as determined by western ligand blotting, with no change in IGFBP-3 immunoreactivity (Guidice et al., 1990). In human seminal fluid, prostate specific antigen (PSA) can function as a potent IGFBP-3 protease (Cohen et al., 1994). The proteolytic cleavage of IGFBP-5 to lower molecular weight forms reduced its affinity for IGF-I (Camacho-Hubner et al., 1992). Phosphorylation of IGFBP-1 (serines 119 and 169) cause a six-fold increase in its affinity for IGF-I (Jones et al., 1993). It has been proposed that, when IGFBP-I is phosphorylated, it has a greater affinity for IGF-I than that of IGF-I receptor, and this favors the binding of IGF-I by the binding protein; when IGFBP-I is dephosphorylated, it has a lower affinity for IGF-I, and IGF-I binding to its receptor is favored.

It is also the emerging consensus that IGFBPs may have IGF-independent functions. IGF binding proteins most likely exert this function through RGD interactions with extracellular matrix and integrins on the cell surface (Jones et al., 1993; Delhanty and Han 1993).

Gene structures. Similar to the protein structures, the genes encoding the six IGFBPs share several common features. First, the localization of the IGFBP gene family in the genome is closely associated with the HOX gene family (Allander et al., 1993; Allander et al., 1994). Both these families localize to four regions in the human: 1). 2q31-2q34 contains IGFBPs-2 and -5, and the HOXD gene cluster (Cannizzaro et al., 1987; Ehrenborg et al., 1991). 2). 7q15-7q12 contains IGFBPs-I and -3, and the HOXA gene cluster (Ehrenborg et

al., 1992). 3). 17q12-17q22 contains IGFBP-4 and the HOXB cluster (Bajalica et al., 1992). 4). chromosome 12 contains IGFBP-6 and the HOXC gene cluster (Shimasaki et al., 1991b; Scott, 1992). Secondly, the genes encoding the IGFBPs have similar structures: the coding regions are divided into four exons; the corresponding exons are similar in size and sequence; all genes have a relatively large first intron; and all the promoters, with the exception of that for the IGFBP-2 gene, contain TATA boxes (Rechler and Brown 1992; Allander et al., 1993; Zhu et al., 1993; Gao et al., 1993).

In summary, the IGFBPs, in all likelihood, have important roles in the IGF axis by virtue of their endocrine, autocrine and paracrine actions, tissue and developmental stage-specific expression, posttranslational modifications and potential ligand-independent actions.

IGFBP-2 Gene and Physiology

IGFBP-2 Gene

Structure. The IGFBP-2 gene and/or complementary DNA has been cloned and sequenced from the human (Binkert et al., 1989; Ehrenborg et al., 1991; Binkert et al., 1992), rat (Brown et al., 1989; Margot et al., 1989; Brown and Rechler, 1990; Kutoh et al., 1993), mouse (Landwehr et al., 1993), cow (Bourner et al., 1992), sheep (Delhanty and Han, 1992) and chicken (Schoen et al., 1995). The IGFBP-2 chromosomal gene structures, in those species that were studied (human, rat, mouse and chicken), are highly conserved. This gene contains 4 exons and 3 introns. Because of the large first intron (23-32 kb), these genes span 28 to 38 kb in length. The size of the third exon is identical among animals, whereas the

other exons and introns are very similar in size, except for exon 4 of the chicken which contains about 800 bp of additional noncoding sequence.

The promoter of the IGFBP-2 gene is GC rich and lacks TATA and CAAT boxes, whereas the promoters of the other IGFBP genes contain TATA boxes. The promoter region of IGFBP-2 gene is highly conserved across species and contains at least 3 G/C boxes. In the rat, it has been shown that three clustered G/C boxes bind SP-1 transcription factor and are required for efficient transcriptional initiation of this gene (Boisclair et al.,1993). In the human, the transcriptional start site was mapped to -113 ± 2 bp relative to the translational start codon ATG (Binkert et al., 1992). In the rat, the transcriptional start site was first placed at -88 nt using primer extension with a primer from +4 to -22 nt. However, it was mapped to -151 nt using primer extension with primers from -65 to -104 nt and -76 to -99 nt. The latter position was confirmed by RNase protection assay. Therefore, the transcriptional start site was reported to correspond to -151 nt (Brown and Rechler, 1990). In 1993, however, Schwander and colleagues reported a transcriptional start site for the rat IGFBP-2 gene at -90 nt as identified using primer extension and S1 nuclease protection assays (Kutoh et al., 1993). Using reverse ligation-PCR (RLPCR), Boisclair and Brown (1995) provided corroborative evidence that transcription of the rat IGFBP-2 gene initiates at a cluster extending from -86 to -90 nt (relative to the ATG, +1), instead of the -151 nt. In the mouse, this start site was mapped to -85 nt (Landwehr et al., 1993), which corresponds to -84 nt of the rat IGFBP-2 gene. Nevertheless, this highly G/C rich promoter and exon may exhibit a secondary structure at the mRNA cap site, which may make it difficult to denature and reverse-transcribe IGFBP-2 cDNA at the 5' end. In addition, promoters lacking TATA-motifs

can exhibit multiple transcriptional initiation sites (for reviews see McKeon et al., 1990; Lu et al., 1994).

Mammalian IGFBP-2 mRNAs are usually in the size range of ~1.5 -1.6 kb. However in human fetal liver, HepG2 and embryonic liver cell lines, a 4.4 kb mRNA was also found (Zapf et al., 1990; Badinga, L. unpublished data). It is not clear why these human cells have a different size of IGFBP-2 transcript. In the chicken, IGFBP-2 mRNA is 2.3 kb in length because of a larger 3'-untranslated region (Schoen et al., 1995).

Localization. In the human, this gene has been localized to chromosome 2q33-34 (Ehrenborg et al., 1991), where it is somewhat close to a cluster of homeobox genes at 2q31-2q37 which are active in the regulation of development (Acampora et al., 1989), a gene encoding cAMP response element binding protein 1 (CREB1) at 2q32.3-2q34 (Taylor et al., 1989), and a gene encoding inhibin α at 2q33-2qter (Barton et al., 1989). Moreover, human IGFBP-2 and IGFBP-5 genes are closely linked in a tail to tail orientation and are about 20-40 kb apart (Allander et al., 1994). Similar to the human, the mouse IGFBP-2 and IGFBP-5 gene are linked in tail-to-tail arrangement separated by only 5 kb, and co-localized to chromosome 1 (Kou et al., 1994). Interestingly, IGFBP-1 and IGFBP-3 genes were also found in a tail-to-tail arrangement in the human (Ehrenborg et al., 1992) and mouse (Kou K., 1994). These gene pairs may prove useful as models to study long-range chromatin structure involved in genetic regulation.

IGFBP-2 Gene Expression and Regulation

General. Several studies showed that IGFBP-2 gene expression is controlled, at least in part, at the transcriptional level (Babajko et al., 1993; Ooi et al., 1993), as transcriptional

rate and steady state RNA levels were closely correlated and increases in mRNA abundance were found without correspondent changes in mRNA stability (Mouhieddine et al., 1996). Unlike the IGF-II and IGF-II receptor genes, the IGFBP-2 gene is normally expressed from both alleles and is not imprinted (Wood et al., 1994).

Developmental changes. IGFBP-2 gene expression is developmentally regulated in those species where examined. The level of IGFBP-2 mRNA is higher in liver, kidney, intestine and lung of fetal rats at late gestation than in these same tissues of adult rats (Brown et al., 1989). IGFBP-2 mRNA is expressed at high levels in the livers of term-gestation and early neonatal rats, and was greatly reduced in Day 21 postnatal and adult rats (Ooi et al., 1993; Babajko et al., 1993). IGFBP-2 in chicken embryo serum increased progressively between embryonic stages E10 to E22 (Yang et al., 1993). In the pig, hepatic IGF-II and IGFBP-2 mRNA levels were higher at late gestation and birth than at 21 and 49 days of age (Kampman et al., 1993). Levels of IGFBP-2 in the fetal pig circulation increased from fetal Days 45 to 110 (McCusker et al., 1988). IGFBP-2 was 2- to 3-fold more abundant in fetal serum than in postnatal serum (Lee et al., 1991), while IGFBP-2 levels in pregnant gilt serum were unaltered during pregnancy (Lee et al., 1993). In the ovine fetus, expression of the IGFBP-2 gene was ubiquitous in tissues before 80 days of gestation, whereas this became restricted to the liver, kidney and choroid plexus after Day 80 of gestation (Delhanty and Han, 1993a).

In the human ovary, the follicular fluid of dominant follicles contains lower levels of IGFBP-2 than subordinate or atretic follicles (Schuller et al., 1993; San Roman and Magoffin, 1992). Similarly, levels of IGFBP-2 in dominant follicles were lower than in

subordinate follicles of cattle (Thatcher et al., 1996). In porcine ovarian follicles, IGFBP-2 mRNA level was positively correlated with day of cycle and follicle diameter (Samaras et al., 1993)

IGFBP-2 mRNA abundance in the livers of lactating ewes is markedly increased compared to that for pregnant ewes, whereas in mammary glands IGFBP-2 mRNA expression was lower in lactating than pregnant ewes (Klempt et al., 1993).

Tissue specific expression. IGFBP-2 gene expression appears to be somewhat tissue restricted. It has been shown that the IGFBP-2 gene is expressed in certain tissues including the liver (Margot et al., 1989), ovary (Samaras et al., 1993), testis (Lin et al., 1993a), mammary gland (Simmen et al., 1992), pituitary (Bach and Bondy, 1992; Michels et al., 1993), bone (Schmid et al., 1992), lymphocytes (Neely et al., 1991; Nyman and Pekonen, 1993) and endothelial cells (Moser et al., 1992). IGFBP-2 mRNA is also detectable in adipose tissue of mid-pregnant gilts (Simmen et al., 1992). In rat intestinal epithelial cells, IGFBP-2 is the major IGFBP produced (Park et al., 1992), whereas in the intestine of mid-pregnant gilts, IGFBP-2 is not highly expressed (Simmen et al., 1992). In post-implantation rat embryos, IGFBP-2 is expressed in cell populations that are rapidly dividing or in regions that direct the growth and differentiation of neighboring cells and tissues (Wood et al., 1992; Streck et al., 1992; Green et al., 1994). Studies of the porcine ovary showed that IGFBP-2 mRNA was most abundant in granulosa cells, lower in theca cells, and lowest in luteal cells. However, another study found that IGFBP-2 was not detectable in the medium of rat granulosa cells (Liu et al., 1993b). In mid-pregnant gilts, the IGFBP-2 gene is highly

expressed in uterus, liver and mammary gland, whereas there is little or no expression in heart, muscle, pancreas and skin (Simmen et al., 1992).

In cancer cells. IGFBP-2 is expressed by some ovarian carcinoma cell lines, such as PEO4, EFO-21, MFO-35, MFO-36, while not expressed in other ovarian carcinoma cell lines, such as EFO-27 (Krywicki et al., 1993; Hofmann et al., 1994). IGFBP-2 mRNA abundance was 2- to 30-fold higher in malignant ovarian tumors than in benign ovarian neoplasms (Kanety et al., 1996). The human breast cancer cell lines MCF-7 and BT-20 synthesize and secrete this protein into the medium, whereas the MDA-MB-231 cell line does the not express this particular IGFBP (Kim et al., 1991).

Metabolic effects. Fasting can induce IGFBP-2 mRNA abundance in the livers of adult rats (Orlowski et al., 1990; Ooi et al, 1993). This induction can be reversed by refeeding (Ooi et al., 1993). Starvation increased the fetal plasma levels of ovine IGFBP-2 (Gallaher et al., 1992). Liver IGFBP-2 mRNA levels were elevated in intrauterine growth-retarded (IUGR) piglets at day 90 of gestation and at birth (Kampman et al., 1993). However, another study indicated that maternal fasting did not alter the abundance of IGFBP-2 mRNA in fetal rat liver (Straus et al., 1991). In dairy cows, the negative nutrient balance during early lactation increased the serum concentration of IGFBP-2 (Vicini et al., 1991). A study (Pucilowska et al., 1993) on measurement of IGFs and binding proteins before and after 21 days of refeeding of 22 undernourished Bangladesh children (2-4 years of age), showed that the IGFBP-2 serum concentration before refeeding was twice that in controls and became normalized after refeeding of a high protein diet, but remained high in these individuls fed

the normal protein diet, suggesting that circulating IGFBP-2 levels are sensitive to dietary protein intake.

IGFBP-2 mRNA in livers of diabetic rats was increased over that for normal animals (Ooi et al., 1992). This increase was, however, reversed by treatment with insulin.

Hormonal effects. Using cultured human endometrial stromal cells, Giudice and colleagues have shown that the combination of estrogen plus progesterone increased IGFBP-2 secretion by 10 to 15-fold and that progesterone alone stimulated IGFBP-2 mRNA steady-state levels by 12 to 15-fold (Giudice et al., 1991b). This stimulation by progesterone was blocked by the receptor antagonist, RU 486. EGF plus progesterone also had stimulatory effects on IGFBP-2 gene expression in these same cells (Giudice et al., 1992). In the anterior pituitary of ovariectomized rats, estrogen increased IGFBP-2 mRNA abundance (Michels et al., 1993). In ovarian cancer cells, however, estrogen minimally depressed IGFBP-2 mRNA abundance (Krywicki et al., 1993). Similarly estrogen did not influence IGFBP-2 mRNA abundance in the human breast cancer cell line MCF-7 (Kim et al., 1991). In this same study, it was shown that α-difluoromethylornithine (DFMO) an inhibitor of polyamines (PA), inhibited IGFBP-2 gene expression in the BT-20 and MDA-MB-231 cell lines by an as yet unknown mechanism.

In alveolar epithelial cells, TGF-β1 was a potent stimulator of IGFBP-2 gene expression (Cazals et al., 1994). In rat hepatocytes, retinoic acid stimulated IGFBP-2 gene expression, and insulin blocked both the basal and retinoic acid-induced IGFBP-2 expression (Schmid et al., 1992). A most recent study has shown that retinoic acid (RA) inhibits proliferation of bovine mammary epithelial cells and increases levels of IGFBP-2 in

conditioned medium and in plasma membrane preparations (Woodward et al., 1996). However, such effects of retinoic acid were not observed in osteoblasts, suggesting that this regulation is tissue-specific (Schmid et al., 1992).

In rat leydig cells, the expression of both IGFBP-2 and its mRNA were decreased by hCG in a dose-dependent manner (Wang et al., 1994). Treatment with 10 ng/ml hCG reduced by 32% the transcription rate of the IGFBP-2 gene, whereas the half-life of the mRNA remained unchanged. Forskolin decreased IGFBP-2 mRNA abundance and protein synthesis in MDBK bovine kidney epithelial cells (Cohick et al., 1991). Phytohemagglutinin (PHA), in contrast, increased IGFBP-2 mRNA production in human lymphocytes (Nyman and Pekonen, 1993)

Treatment with bovine somatotropin decreased the serum concentration of IGFBP-2 in cows at early lactation, late lactation and the dry period (Vicini et al., 1991). Clemmons and colleagues found that the serum concentration of IGFBP-2 is usually elevated in patients with Growth Hormone deficiency (GHD), whereas IGF-I and IGFBP-3 concentrations are lower than normal (Clemmons et al., 1991; Smith et al., 1993). However, the ratio of IGFBP-2/IGF-I failed to constitute a reliable test for diagnosis or exclusion of GHD in all short children. Moreover, it was reported that Growth Hormone deficiency in 'little' mice does not affect the serum level of IGFBP-2 (Donahue and Beamer, 1992). Using primary cultures of hepatocytes, Schwander and colleagues demonstrated that insulin was a negative regulator of hepatic IGFBP-2 mRNA expression, whereas Growth Hormone had no effect (Boni-Schnetzler et al., 1990).

A developmental switch from high expression of IGFBP-2 in fetal rats to low expression in adult rats led investigators to examine the effects of thyroid hormone on the expression of this gene. It was reported that hypothyroid pups continue to manifest high levels of serum IGFBP-2 and IGFBP-2 mRNA in liver up to 19 Days of age, and that treatment with thyroid hormone decreased this high expression, thereby indicating that thyroid hormone may inhibit IGFBP-2 gene activity (Nanto-Salonen et al., 1991).

Recent studies showed that glucocorticoid (GC) rapidly decreased DNA synthesis and proliferation of lung alveolar epithelial cells, which was associated with accumulation of IGFBP-2 in the culture medium, and increases of IGFBP-2 mRNA in these cells (Mouhieddine et al., 1996). Transfection using a 1.4 kb promoter region of the rat IGFBP-2 gene demonstrated that this region can respond to GC treatment with increased luciferase reporter gene activity.

Summary of IGFBP-2 gene expression and regulation. 1). Although IGFBP-2 is expressed in a number of tissues, it tends to be more expressed in epithelia and the supporting stroma cells. 2). Since this gene often is up-regulated during certain physiological conditions such as fasting, starvation, negative nutrient balance, cancer, and pregnancy, IGFBP-2 may have some protective (homeostatic) function. 3). Estrogen, progesterone, TGF-β, phorbol ester, GC and retinoic acid appear to be stimulatory, whereas GH, insulin, thyroid hormone and hCG, appear to be inhibitory for this gene's expression. 4). Control of expression of this gene appears to be complicated and divergent between tissues and across species.

Physiology of IGFBP-2

Protein. IGFBP-2 has been purified from conditional medium of the buffalo rat liver cell line (BRL 3a) (Mottola et al., 1986) and the Madin-Darby Bovine Kidney epithelial cell line (MDBK) (Szabo et al., 1988). Therefore, this protein was called the BRL3A cell line-derived IGF BP, MDBK cell line-derived IGF BP or IBP-2 (Drop et al., 1992). The new nomenclature has been used since 1991, as mandated by participants in the second International IGF/Somatomedin Symposium in San Francisco. The mature IGFBP-2 in human (Binkert et al., 1989), rat (Brown et al., 1989; Margot et al., 1989), mouse (Landwehr et al., 1993), cow (Bourner et al., 1992), sheep (Delhanty and Han, 1992) and chicken (Schoen et al., 1995) has 289, 270, 289, 284, 284 and 275 amino acids and 31 kDa, 29.5 kDa, 34 kDa, 34 kDa, 31 kDa and 33.5 kDa molecular weights, respectively. This protein from all species studied contains 18 conserved cysteine residues and an RGD sequence. Mutations of these cysteines influence the affinity of IGF binding (Coulter et al., 1995). Infusion of pure IGFBP-2 in rats demonstrated that the half-life for this protein in the bloodstream was 144 ± 32 min (Young et al., 1992).

Functions. IGFBP-2 purified from MDBK cells had higher affinity for IGF-II than IGF-I (Bourner et al., 1992). IGF-I binds to IGFBPs-3 and -1 with higher affinity than to IGFBP-2 (McCusker et al., 1991). IGFBP-2 protein enhanced the DNA synthesis response of porcine aortic smooth muscle cells to the IGF-I present in platelet-poor plasma (ppp). However, in serum-free medium, it blocked the stimulation by IGF-I. Similarly, an early study showed that MDBK IGFBP-2 inhibited the ability of IGF-II to stimulate DNA synthesis, and protein accumulation in chick embryo fibroblasts, while a lesser effect on IGF-

I was observed (Ross et al., 1989). Bovine IGFBP-2 inhibited binding of IGF-I to the cell surfaces of human fetal fibroblasts (GM10 cells) and porcine smooth muscle cells (McCusker et al., 1991). Human recombinant IGFBP-2 with a single amino acid mutation ([Cys^{281}]rhIGFBP-2) has been expressed and purified from the conditioned medium of a clonal Chinese hamster ovary cell line (Feyen et al., 1991). This protein inhibited IGF-I-stimulated cell proliferation in a dose-dependent manner, whereas it had no effect on insulin stimulated cell proliferation. In addition, IGFBP-2 inhibited both basal and IGF-I stimulated bone collagen synthesis. It has been reported that soluble IGFBP-2 inhibited the binding of IGF-I and IGF-II to SCLC and NSCLC cells and inhibited IGF-stimulated DNA synthesis in NSCLC cells (Reeve et al., 1993).

Accumulating evidence indicates that IGFBP-2 may have IGF independent functions. In vitro studies suggested that IGFBP-2 binds to cell membrane proteoglycans through its glycosaminoglycan binding domain (PKKLRP) (Russo et al., 1996). It has been demonstrated that IGFBP-2 stimulates Growth Hormone receptor binding of Growth Hormone and mRNA abundance of Growth Hormone receptor (Slootweg et al., 1995). Inhibition of endogenous IGFBP-2, by transfection of the cDNA antisense expression construct, stimulates proliferation of intestinal epthelial cell lines (Corkins et al., 1995).

Inactivation of one or both alleles of the IGFBP-2 gene in mice did not affect pre-natal and post-natal growth, whereas in these animals the serum concentrations of IGFBP-3 and IGFBP-1 were increased (Wood et al., 1994). These data support the concept that IGFBPs may have overlapping functions and be compensatory to some extent.

Collectively, IGFBP-2 may have several potential functions including inhibition and/or modulation of IGF actions in soluble form and in association with cell membranes or IGF-independent functions. However, since only few functional studies of IGFBP-2 have been conducted, the function of IGFBP-2 remains relatively undefined.

Uterine IGF System

The uterus is an important reproductive organ with many physiological functions in pregnancy and fetal growth and development. These functions which include uterine gene expression, protein synthesis and secretion, maternal-fetal nutrient exchange and utero-ovarian interactions are controlled not only by systemic hormones but also by locally produced factors. Uterine IGFs and their binding proteins are implicated in the control of uterine cell proliferation and differentiation in cyclic and pregnant animals. This family of growth factors together with other hormones, growth factors and cytokines constitutes a complicated and efficient uterine growth control network.

Differential Expression and Distribution in Uterine Tissues

A number of studies have demonstrated that IGFs, IGF receptors and IGFBPs are expressed in uterine tissues and cells (reviewed by Simmen et al., 1995). Expression of this system exhibits tissue and developmental specificity.

IGFs and IGF receptors. In the pig (Simmen et al., 1992; Song et al., 1996), IGF-I and IGF-II genes are expressed in the endometrium and myometrium. Expression of the IGF-I gene is higher in tissues of early than later pregnancy, whereas expression of IGF-II is lower in the tissues of early than later pregnancy. Peak levels of IGF-I mRNA occur on Day 12 of the cycle and pregnancy in pigs. IGF-I receptor numbers are low and do not change with

stage of pregnancy (Simmen et al., 1992). IGF-II receptor expression in pig uterus has not been examined. In the human (Guidice et al., 1993), IGF-I mRNA is primarily expressed in proliferative and early secretory endometrium. Abundant IGF-II mRNAs are expressed in mid-late endometrium and early pregnancy decidua. IGF-I and IGF-I receptor mRNAs are abundantly expressed in secretory endometrium and early pregnant decidua. In the rat (Zhang et al., 1994), both IGF-I and IGF-II mRNAs are expressed in the uterus during the periimplantation period .

IGFBPs. Similar to the IGFs and their receptors, IGFBPs are also differentially expressed in the uterus. In the rat (Girvigian et al., 1994), IGFBP-2 and IGFBP-4 mRNAs were localized in luminal epithelium of endometrium. IGFBP-2 mRNA is more highly expressed during pro- and early estrus than at other stages of the cycle, while IGFBP-4 mRNA is present only at diestrus. IGFBP-3 mRNA is found in stromal cells and is highly expressed on Day 12 of pregnancy. IGFBP-5 and IGFBP-6 mRNAs are maximally expressed in myometrium. Maximal levels of IGFBP-5 mRNA are at estrus, whereas IGFBP-6 mRNA abundance peaks at estrus. IGFBP-1 expression in the rat uterus was detected in one study (Ghahary et al., 1993) , but was undetectable in another study (Girvigian et al., 1994). In the pig, IGFBP-5, and -6 mRNA are expressed in both the endometrium and myometrium (Song et al., 1996). IGFBP-2 is expressed at low levels in endometrium of early pregnancy and at high levels in endometrium of late pregnancy of pigs, with little or no expression in myometrium (Simmen et al., 1992). IGFBP-I is the major IGFBP produced by the endometrium and decidua of the human and baboon, which have a haemochorial placentation (Giudice et al., 1991a, b; Tarantino et al., 1992; Tang et al., 1994). IGFBP-2 and -3 are less

abundantly expressed, although still at significant levels in these tissues. Interestingly, IGFBP-1 gene expression is not detectable in the uterus of the pig, a species which exhibits epitheliochorial placentation (F. A. Simmen, unpublished observations).

Potential Uterine Functions of IGFs

In maternal fetal interactions. One example of how IGFs might be involved in maternal:fetal interactions is the paracrine role of IGF-I to stimulate estrogen production from periimplantation conceptuses in the pig model. During the periimplantation period, the porcine endometrial IGF-1 gene exhibits a peak of expression at Day 12 (Simmen et al., 1992). At this same time, IGF-1 receptors are expressed in endometrium (Hofig et al., 1991) and conceptus (Green et al., 1995). This unique expression pattern is temporally coincident with a transient period of secretion of estrogens from porcine conceptuses. An *in vitro* study showed that treatment with IGF-1 stimulated the abundance of mRNA for P450 aromatase in Day 12 filamentous conceptuses (Green et al., 1995). These data support the model proposed by Simmen et al. (1995). In this model, IGF-1 from endometrium stimulates conceptus aromatase gene expression by binding to IGF-1 receptors on the conceptus. Aromatase serves as a key enzyme in the pathway of estrogen synthesis. Estrogens during this specific physiological condition are known to serve as the biological signals for maternal recognition of pregnancy in pigs (Bazer et al., 1991). Estrogens also may be involved in inducing or reducing uterine expression of genes, such as *c-jun* and *c-fos* (Cicatiello et al., 1993), which in turn probably regulate uterine functions.

In contrast to enhancing embryo development, IGF-I may also be responsible, in part, for early embryo losses (Katagiri et al., 1996). Superovulation increases early embryo losses

and IGF-I levels in uterine luminal fluid (ULF) of the rat. Both normal ULF infused with IGF-I and ULF from superovulated rats impaired embryo development *in vitro*. Anti-IGF-I antibody infusions after superovulation reversed the detrimental effect of superovulation.

Evidence supporting an IGF contribution to embryo losses is from an early study in the pig model (Simmen et al., 1992). During the periimplantation period, expression of IGF-II mRNAs are lower in the endometria from the Meishan breed with low conceptus mortality, than from the Large White breed exhibiting high conceptus mortality. However, in this case, IGF-I mRNA abundance was high in both breeds. IGF effects may parallel the morphogen retinoic acid. The latter molecule is believed to be an embryotoxic factor when transported in larger than normal quantities into the uterine lumen (reviewed by Roberts et al., 1993).

In implantation. A major function of the endometrium is to participate in implantation of the conceptus and support subsequent pregnancy. In the epithelialchorial placental species, such as the pig, the role of luminal and glandular epithelial cells is predominant, whereas in the hemochorial species, in which trophoblast invasion is extensive, differentiation of the stromal compartment with formation of the decidua is characteristic. Temporal expression of IGFs and their binding proteins in the endometrium provides a possibility that they may be important for implantation in these different species. A recent study (Markoff et al., 1995) showed that mouse IGFBP-4 mRNA becomes strongly expressed at each implantation site and extends throughout the decidua, 24 hours after implantation, whereas no expression in the uterine tissue between implantation sites was observed. This anatomically and temporally characteristic expression pattern suggests a physiological role for IGFBP-4 in the implantation process. Similarly in the baboon, IGFBP-I and -2 are the predominant IGFBPs

in the endometrium. During early pregnancy, IGFBP-I is only present in stromal cells that are in intimate contact with the trophoblastic tissue (Tarantino et al., 1992). IGFBP-1 contains an RGD sequence, which is commonly seen on extracellular matrix protein receptors and has been shown to be the recognition site for integrin cell surface receptors (Jones et al., 1993). Since extracellular matrix is important for contact and migration of trophoblast and endometrium (Kliman and Feinberg, 1990), IGFBP-1 may serve as a special extracellular matrix binding protein during the implantation process.

Porcine Uterine IGFBP-2 and Remaining Problems

Uterine IGFBP-2 gene exhibits several interesting expression features (Simmen et al., 1992, Song et al., 1996). First, the expression of this gene is tissue specific. It is restricted in endometrium, while no expression is detected in the myometrium and placenta. In endometrium, glandular and luminal epithelial cells as well as stroma express IGFBP-2. Second, the expression of this gene is developmentally and temporally regulated. In cyclic pigs, endometrial IGFBP-2 expression is high during estrus, and low during diestrus and exhibits a cyclic wave. In endometrium of pregnant pigs, IGFBP-2 expression is low at early pregnancy, increases from periimplantation, and reaches maximal levels at mid-pregnancy. Third, this gene appear to be hormonally regulated. In cyclic pig endometrium, IGFBP-2 expression follows changes in estrogen concentration in the circulation, and is negatively correlated to the systemic changes in progesterone concentration.

IGFBP-2 appears to have a specialized function in the feto-maternal interface. Immunohistochemical localization showed that IGFBP-2 accumulates on the surface of endometrial epithelial cells of pregnancy (Song et al., 1996). This observation implicates

IGFBP-2 in feto-maternal interactions and perhaps as a specific modulator of extracellular matrix protein-cellular interactions through its RGD and/or other sequence motifs.

Based on its novel gene expression pattern and protein localization, a number of interesting questions remain to be investigated: 1). What are the molecular mechanisms, including *cis*- and *trans*-element interactions, DNA methylation, chromatin structure, underlying the temporally-regulated and endometrial-specific transcription of the IGFBP-2 gene? 2). What hormones regulate IGFBP-2 gene expression at the level of transcription? 3) Are there endometrial specific transcription factors? 4) What are the functions of uterine IGFBP-2, especially at the feto-maternal interface? Answers to these questions may be helpful for furthering our understanding of uterine gene expression and protein synthesis during pregnancy.

CHAPTER 3
THE UNIQUE ENDOMETRIAL EXPRESSION AND GENOMIC ORGANIZATION OF THE PORCINE IGFBP-2 GENE

Introduction

The IGF binding proteins (IGFBPs) are a class of six growth-regulatory molecules that exhibit sequence relatedness and the capacity to bind the mitogens IGF-I and IGF-II (reviewed by Rechler, 1993; Jones and Clemmons, 1995). These proteins can associate with cell membranes and extracellular matrix, where they influence IGF:IGF-receptor interactions and possibly exert IGF-independent functions (Jones and Clemmons, 1995). Each IGFBP is encoded for by a separate gene containing a unique set of transcriptional regulatory sequences. Nevertheless, a particular cell-type may synthesize more than one IGFBP (Giudice et al., 1991a, b; Girvigian et al., 1994; Ko et al., 1994b). The biological role(s) of each IGFBP for a given tissue or cell-type remains relatively undefined by virtue of the apparent redundancy in the expression and postulated autocrine/paracrine actions of this protein family.

Previous work documented the temporally regulated expression during pregnancy of mRNAs encoding components of the uterine IGF system (Simmen and Simmen, 1990; Simmen et al., 1990, 1992; Geisert et al., 1991; Tarantino et al., 1992; Green et al., 1995). The nature of the major IGFBP(s) produced by uterine tissues may vary considerably for

mammalian species during pregnancy. In the human, baboon and rat, species which exhibit haemochorial placentation, IGFBP-1 is the major IGFBP synthesized in the endometrium and decidua (Giudice et al., 1991a, b; Tarantino et al., 1992; Tang et al., 1994). IGFBP-2 and -3 are less abundantly expressed in uteri of these species. The pig is characterized by noninvasive, epitheliochorial placentation and has been used by our laboratories to examine the involvement of the uterine IGF system in embryo and conceptus development (reviewed in Simmen et al., 1993; 1995). Pig uterine endometrium maximally expresse mRNAs encoding IGF-I and IGF-II at periimplantation (Day 8-12 post-mating) and post-implantation stages of pregnancy (114-115 days, total length), respectively (Simmen and Simmen, 1990; Simmen et al., 1992). The IGFBP-2 gene exhibits abundant mRNA expression in the porcine endometrium and this is maximal at mid/late-pregnancy (Simmen et al., 1992). In sexually mature, nonpregnant pigs, uterine endometrial IGFBP-2 mRNA abundance varies in parallel with circulating estrogen concentrations and is negatively correlated with serum progesterone concentration (Simmen et al., 1992). It also was shown that IGFBP-2 mRNAs are expressed in uterine luminal epithelial, glandular epithelial and stromal cells, with little or no mRNA in myometrium and placenta. However, immunohistochemical localization studies demonstrated that IGFBP-2 protein is produced and/or accumulated in the luminal and glandular epithelium but not in the endometrial stroma (Song et al., 1996).

In order to provide the requisite foundation for elucidating the molecular mechanisms underlying this interestinting uterine gene expression phenotype, the pig IGFBP-2 chromosomal gene was cloned and characterized in this chapter.

Materials and Methods

Animals

Gilts were monitored twice daily for onset of estrous activity. Gilts exhibiting two consecutive estrous cycles of normal duration (18-22 days) were mated at estrus with boars and again 12 and 24 h later. The day of onset of estrus was defined as Day 0 of pregnancy. Animals were sacrificed at the University abattoir on the indicated days of pregnancy. Reproductive tracts were removed, immersed in ice and trimmed from the mesometrium. Endometria, myometria and placentae were obtained by dissection as previously described (Simmen et al., 1990; Ko et al., 1994a). Animal use protocols were approved by the University of Florida Institutional Animal Care and Use Committee.

Northern Hybridization

Procedures used for extraction of total cellular RNA, isolation of poly(A)$^+$-RNA, formaldehyde-agarose gel electrophoresis, blotting of RNA to nylon membranes, preparation of gel-purified, ^{32}P-labeled IGFBP cDNA fragment and blot-hybridization were described previously (Lee et al., 1993a, b; Ko et al., 1994b). Cloned cDNA insert used as probe encoded rat IGFBP-2 (Brown et al., 1989). Sizes of IGFBP-2 mRNAs were calculated based on the migration positions of the 18S and 28S ribosomal RNAs (2 and 5 kb, respectively).

Isolation and Mapping of Pig IGFBP-2 Cosmid Clones

A library of porcine genomic DNA fragments (derived from one male pig) cloned in the cosmid vector pWE15 was obtained from Clontech Laboratories, Inc. (Palo Alto, CA). Cosmids were screened by colony-hybridization with a rat IGFBP-2 cDNA fragment (*Sac*I X *Sma*I, 530 base pairs; Brown et al., 1989) labeled with ^{32}P-dCTP by nick-translation. Five

positive clones were obtained after several rounds of re-screening and three of these underwent detailed restriction analysis. The relative locations of restriction-endonuclease cleavage sites in cosmid DNA inserts were determined using the Cosmid Mapping System (Gibco-BRL, Inc., Gaithersburg, MD).

DNA Subcloning and Sequencing

Exons were preliminarily assigned to restriction fragments of cosmid clones by Southern blot-hybridization with end-labeled, exon-specific oligodeoxynucleotide probes designed by computer analysis of conserved mammalian IGFBP-2 cDNA sequences. Restriction fragments that were positive by Southern blot were isolated from cosmid clones by agarose gel electrophoresis and subcloned in pGEM-3Z (Promega Corp., Madison, WI). DNA sequences of plasmid subclones were determined using the Sanger dideoxy procedure, Sequenase (USB, Amersham Life Science, Cleveland, OH) and a primer-walking strategy. Oligodeoxynucleotide primers were synthesized by the DNA Synthesis Core of the Interdisciplinary Center for Biotechnology Research at the University of Florida. All DNA sequences were confirmed on both strands and in some cases, sequencing reactions were modified (e.g., by altering the temperature of denaturation, extension and/or termination steps or by inclusion of deoxyinosine triphosphate) in order to sequence through problematic G/C-rich areas. DNA sequences were compiled and analyzed using the Sequence Analysis Software Package of the Genetics Computer Group, Inc. (GCG Package, Version 7, 1991). The genomic sequences encompassing pig IGFBP-2 exons 1, 2-3 and 4 have GenBank Accession Numbers U21117, U21118 and U21119, respectively.

Analysis of Intron 3

A fragment encompassing all of intron 3 was amplified from endometrial DNA (of one pig) by high fidelity PCR (Expand High Fidelity PCR System, Boehringer Mannheim, Indianapolis, IN). The upstream primer was 5'- TGACAAGCATGGCCTGTACAACCTC-3' (within exon 3) and the downstream primer was 5'-ACGCTGCCCATTCAGAGACATC TTG-3' (within exon 4). The PCR fragment was cloned using the TA cloning Kit (Invitrogen, San Diego, CA) and analyzed by restriction endonuclease-digestion and partial DNA sequencing.

Primer Extension

The procedure used for the primer extension assay was described previously (Simmen et al., 1989). Briefly, the ^{32}P-end labeled primer 5'-GGCAGCATGTTGGCG-3' was coprecipitated with poly(A)$^+$ RNA, total cellular RNA or yeast RNA (as negative control), and the precipitate dissolved in 30 µl of hybridization solution (50% formamide, 40 mM Tris-HCl, pH 7.5, 400 mM NaCl, and 1 mM EDTA). This solution was incubated at 65 °C for 30' and then at 42 °C for 16 hours. Nucleic acids were precipitated and dissolved in extension buffer (50 mM Tris-HCl, pH 8.0, 50 mM KCl, 5 mM $MgCl_2$, 5 mM DTT, 4 mM dNTPs and 1U RNase inhibitor). The extension reaction was initiated by addition of 40 U AMV reverse transcriptase (Invitrogen, San Diego, CA), and the samples were incubated at 42 °C for 90 min. Extended DNA products were separated on a 6% denaturing, polyacrylamide gel. Sequencing reactions using the same primer (without ^{32}P-label) and plasmid H6 (see Results) as template were electrophoresed on the gel as size markers.

S1-Nuclease Protection Assay

Sma-LUCe plasmid DNA (see Chapter 4, Materials and Methods), which contains an IGFBP-2 genomic fragment spanning from -305 to +73 relative to the ATG and 3 bp of *Hin*dIII linker sequence, was linearized at the *Sma*I site and used as template for synthesis of labeled antisense probe by asymmetric PCR. The oligonucleotide primer used in PCR was 5'-GGCAGCATGTTGGCG-3'. The ^{32}P-labeled, single-stranded probe was purified by denaturing polyacrylamide gel electrophoresis. Hybridization of probe to RNA and subsequent treatment with S1 nuclease was performed using reagents provided in the S1-Assay kit (Ambion Inc., Austin, Texas). The products of the S1-nuclease protection assay were separated on 6% denaturing, polyacrylamide gels.

RNase Protection Assay

The plasmid H6 was cleaved with *Not*I at position +73 relative to the ATG initiation codon. The adhesive ends were made blunt-ended by use of the Klenow fragment of DNA polymerase I. The DNA was ligated to a synthetic *Hin*dIII linker (Cat. # 2172, Promega Corp., Madison, WI) and then was cleaved with *Hin*dIII. A fragment, spanning from -1397 to +73 relative to the ATG, and containing 3 bp of *Hin*dIII linker sequence at the 3'-end was isolated from an agarose gel and ligated to pGEM-3Z plasmid (Promega Corp., Madison, WI) previously linearized at the *Hin*dIII site. The resultant plasmid in which the SP6 promoter is adjacent to the 5'-end of the insert was designated BP2-19. The plasmid in which the SP6 promoter is adjacent to the 3'-end of the insert was designated BP2-18. Plasmid BP2-18 was then cleaved with *Sma*I. The fragment containing the SP6 promoter and the insert, spanning from +73 to -305, was purified by agarose gel electrophoresis and DEAE

paper, and used as template for synthesis of antisense RNA probe for RNase protection assay. The ^{32}P-labeled probe was synthesized using the MAXIscript™ SP6/T7 *in vitro* transcription Kit (Ambion Inc., Austin, Texas). RNase protection assay was performed using the HybSpeed™ RPA Kit (Ambion Inc., Austin, Texas). Products of the RNase protection assay were resolved on 6% denaturing, polyacrylamide gels.

Hela Cell *In Vitro* Transcription System

Plasmid BP2-19 was cleaved with *Sma*I + *Hin*dIII. The fragment spanning from the *Sma*I to *Hin*dIII sites was purified by agarose gel electrophoresis and DEAE paper, and used as template for *in vitro* transcription using the Hela Cell Extract Transcription System (Promega Corp., Madison, WI). The ^{32}P-labeled transcripts were separated on 6% denaturing, polyacrylamide gels.

Results

IGFBP-2 mRNAs in Pregnant Pig Endometrium

Previously, it was reported that IGFBP-2 mRNA abundance (i.e., steady-state level) was highly induced in the endometrium of pigs after the implantation period as analyzed by the RNA dot blot-hybridization technique (Simmen et al., 1992). In order to confirm this gene expression pattern, Northern blot-hybridization for IGFBP-2 mRNA was performed. The pig endometrial IGFBP-2 transcript (~1.6 kb in length) was confirmed to be of higher relative abundance after implantation (i.e., Days 30-90) than prior to or during the periimplantation period (Days 8-12) (Figure 3-1).

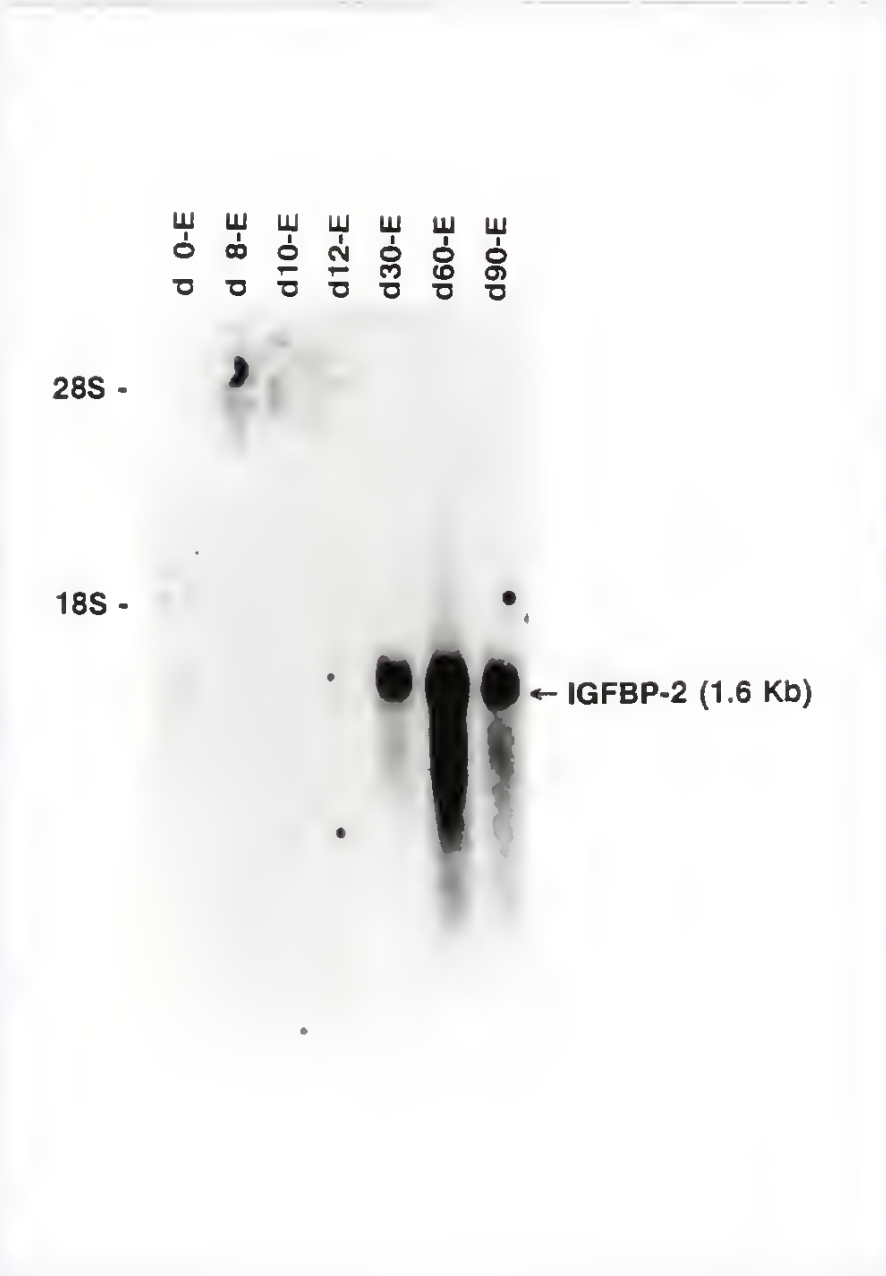


Figure 3-1. Northern analysis of IGFBP-2 mRNA in pregnant pig uterus. Thirty μg total cellular RNA from pig endometrium at Days 0, 8, 10, 12, 30, 60 and 90 of pregnancy was subjected to Northern analysis with ^{32}P-labeled rat IGFBP-2 cDNA fragment. Gestation in the pig is 114-115 days.

Characterization of the Pig IGFBP-2 Chromosomal Locus

The above and previous studies identified several unique characteristics of the uterine-expressed IGFBP-2 gene. Of particular interest were: I) the marked induction in IGFBP-2 mRNA accumulation with later stages of pregnancy, and 2) the endometrial (as opposed to myometrial) specificity of expression of this gene. Therefore, as a first step to identifying the underlying molecular mechanism responsible for this uterine IGFBP gene phenotype, the porcine IGFBP-2 locus was cloned and characterized. Three cosmids (designated 6-I, 4-9 and 9-7) that collectively spanned this gene were isolated and characterized by restriction endonuclease mapping and Southern blot-hybridization using rat cDNA and synthetic oligodeoxyribonucleotide probes (Figure 3-2). Appropriate plasmid subclones were constructed from these cosmids and the four exons were then identified first, by blot-hybridization with consensus exon-specific oligonucleotide probes and subsequently, by DNA sequencing (Figure 3-3). The intron-exon junctions and putative polyadenylation signal were found to be highly conserved across the IGFBP-2 genes of species where characterized (Table 3-1). In addition, the complete cDNA (~1.4 Kb in length) defined from sequencing analysis of the four genomic exons was in excellent agreement (upon addition of 150-200 nucleotides of poly A) with estimates of length of the corresponding endometrial mRNA (above) and encoded an open reading frame for porcine IGFBP-2 that exhibited high identity with IGFBP-2 proteins from other species (Figure 3-4 and Table 3-2). Comparisons of IGFBP-2 exonic sequences across mammals indicated differential degrees of sequence conservation between the four exons (order of conservation: exons 3 > 4 > 2 > I).

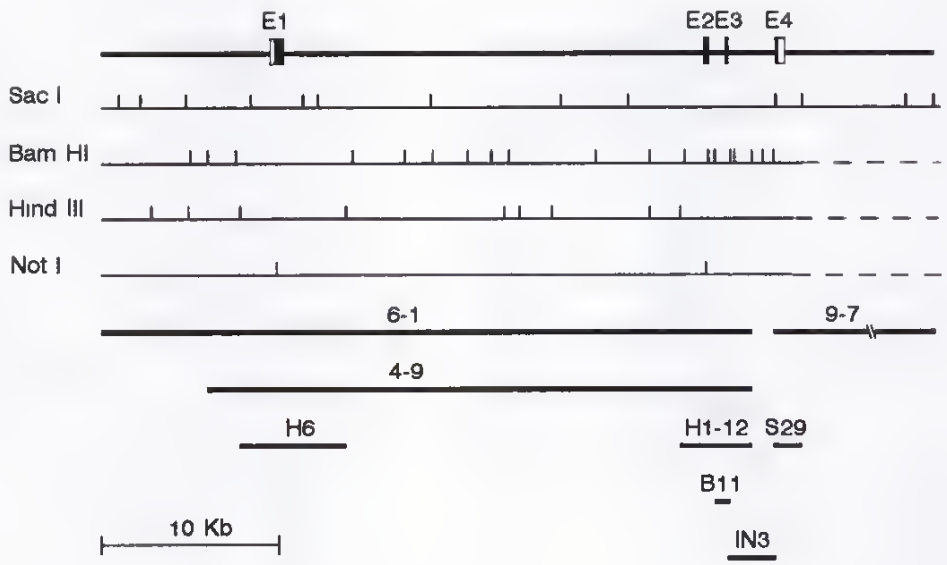


Figure 3-2. Structural organization of the porcine IGFBP-2 chromosomal locus. Cosmids 6-1 and 4-9 overlap exon (E) 1, E2 and E3. Cosmid 9-7 contains E4 and a large segment of DNA flanking the 3' side of E4. Plasmids H6 and H1-12 (Hind III fragments, ~6 kb, derived from Cosmid 6-1,) were subjected to dideoxy sequencing to obtain the DNA sequences of E1, E2 and intron 2. Plasmid B11 (Bam H1 fragment, ~4 Kb, derived from cosmid 4-9) was analyzed to obtain sequence of E3. Plasmid S29 (Sac 1 fragment, ~1.5Kb, derived from cosmid 9-7) was analyzed to obtain the sequence of E4. Plasmid IN3 contains the entire intron 3 fragment which was generated from pig genomic DNA by PCR. Lengths of E1, E2, E3 and E4 are ~527 bp, 227 bp, 141 bp and 528 bp, respectively. Introns 1, 2 and 3 are ~24 Kb, 746 bp and 2.6 Kb, respectively.

Figure 3-3. DNA sequence and deduced amino acid sequence of porcine IGFBP-2 chromosomal gene and protein, respectively. Shown are the sequences of ~1.4 Kb of upstream flanking DNA, exon 1, exon 2, all of intron 2, exon 3 and exon 4. The sites of transcriptional initiation and the polyadenylation signal are capitalized and underlined, respectively. Intron sequences and sequences upstream of the translational initiation codon are depicted in lower case letters.

A. Exon 1

```
        gcttcaatttcaattctgatcccacccctgaaaccccatcacctttcta  50
        tctaaatgcgtgtctcctcctcaaagcctctggcaatctgttgttggttc
        attacaccaaactctcccccaaatggttgttcttgtccatttttgaagat
        cagaaacttgccctcctgggacatccagctcaccttcaaagccaggtagc
        cacctgacaaaagctgcttcagtaaagtcttgaagttggaaagagctgga  250
        gtgcagggccctggcgggggaagggtggcacagaggtattgagaaatta
        tacctggtctggaggcagcttaatggcccacccccaccaagagaatctc
        tctgacaaggatctttgttagggtcacaacccagggagatgggtgataat
        taaagtggggtccagcctggaatttctgggagctgggaacagcttggatc
        aatgattcagtaaatcaaacagaagaataaagggaggccattcttgttca  500
        cctccatttactgaggagagctccaaaggacctaacctcagggcattctc
        ctacacctggaaagctgatctgacctctttaagatccctgaagcagggtg
        ttctggggtctatcctgcagcaaatgtctggagatctgtgggtgggggcc
        gggcccccaagagattaattttctggggagggagcagactaggggagagg
        aagggagcaggtttctcagtgtccagaaactgcatttcaggtacttacat  750
        gttaccagatgctcccagggttgggaaattgcaatggtccctaagagggg
        ctattcagggtacgctggtctagaggatgtgaacagcctccagactccta
        agtcattgtcccaggcagcagtgagtgaaaacccgaggatggaggcagtt
        gcagggagaggactgaaacctacttgaaggtcatgcctgacgtcctttgc
        cggccacatgggaagcgcgcaaacgaagtgcttccgaattgaaccgaaaa  1000
        caggagacaaaagcacccactcttgtccagtcccgcgccaatcttactca
        aacccccaaattattctcatcctccgggagtcagggagcccggggtagaa
        gtgcgtggagggacacagaccggagggagttaggggaccccccgcagcag
        gcgaggaagtggggatgggcgagtgggcgtgcgcgcacgtagaggcccgc
        gcgcgggagtgtcgggggaaggggtggtctccaaaaggggagggagga  1250
        aggcaggggcggggagaagcagggccctttaggacccggctgcggcggc
        gggggagGagaaagaagccgaggaggcggctcccgcgctcgaagggccgt
        gccacctgcccgctcgctcgccgccgtcgcgctcaccgccgccaacATGC
1                                                     MetL

        TGCCGAGACTGGGCGGCACCGCGCTGTCGTTGCTGCCGCTGCTGCTGCTG
3       euProArgLeuGlyGlyThrAlaLeuSerLeuLeuProLeuLeuLeuLeu

        CTGCTGGGCACGGGCGGCCGCGGGGCGCGCCGAGGTGCTGTTCCGCTG  1500
19      LeuLeuGlyThrGlyGlyArgGlyAlaArgAlaGluValLeuPheArgCy

        CCCGCCCTGCACGCCCGAGAGCCTGGCCGCCTGCAGGCCCCCTCCCGCCG
36      sProProCysThrProGluSerLeuAlaAlaCysArgProProProAlaA

        CGCCGCCCTCCGCCGGGGCCGGGCCGGCTGGCGACTCCCGCGCGCCCTGC
53      laProProSerAlaGlyAlaGlyProAlaGlyAspSerArgAlaProCys

        GACCTTGTCCGGGAGCCGGGCTGCGGCTGCTGCTCTGTGTGCGCGCGGCT
69      GluLeuValArgGluProGlyCysGlyCysCysSerValCysAlaArgLe

        GGAGGGCGAGCGGTGCGGCGTGTACACCCCGCGCTGCGCCCAGGGGCTGC
86      uGluGlyGluArgCysGlyValTyrThrProArgCysAlaGlnGlyLeuA

        GCTGCTATCCCCACCCGGGCTCCGAGCTGCCCCTGCAGGCGCTGGTCCTG  1750
103     rgCysTyrProHisProGlySerGluLeuProLeuGlnAlaLeuValLeu

        GGCGAGGGCACTTGCGAAAAGCGCCGCGATGCCGAGTACGGCGCCAGCCC
119     GlyGluGlyThrCysGluLysArgArgAspAlaGluTyrGlyAlaSerPr

        CGAGCAGGTTGCAGgtaacgccggctaggacaactagttcgcagaaactt
136     oGluGlnValAla

        ggagggcaac
```

Figure 3-3 -- continued

B. Exon 2-Exon 3

```
        acctgcagctcttccttgcttgctctttgcagACAATGGCGACGATGCTG  50
140                                     AspAsnGlyAspAspAlaG

        AGGGAGGCCTGGTTGAGAACCACGTGGATGGGAACGTGAACTTGTTGGGA
147     luGlyGlyLeuValGluAsnHisValAspGlyAsnValAsnLeuLeuGly

        GGTACAGGCGGTGCTGGCCGGAAGCCCCTCAAGTCAGGCATCAAGGAGCT
163     GlyThrGlyGlyAlaGlyArgLysProLeuLysSerGlyMetLysGluLe

        GGCTGTGTTCCGGGAGAAAGTCACGGAGCAGCACCGGCAGATGGGCAAGG
180     uAlaValPheArgGluLysValThrGluGlnHisArgGlnMetGlyLysG

        GTGGCAAGCATCACCTCGGCCTGGAGGAGCCCAAGAAGCTGCGGCCGCCG  250
197     lyGlyLysHisHisLeuGlyLeuGluGluProLysLysLeuArgProPro

        CCTGCCAGGgtcagagcaggggttgggtctgattggaggggtgtcccagc
213     ProAlaArg

        atgtgggggtggaagggagtgggcgccccggtgaggtccatgtcttgtg
        tggcctgcaggcaaagcactttcctttctggctctgccactaacattcag
        tgaccttcaggcccatcagtatctcttatgggggctttagcctctttggc
        tttgaaatgggctgagattatgggttcttgaagtcccttccttctaaaac  500
        cttcacatttctccttggctttgggacacaggccaaacactgggtgctag
        agatgaaggtggatcccctggaacttgctcacccttgtcatacgccaccc
        tcactcagctactccttttcctctttccagctcagcattgctgtcttggt
        ctccaccttggttggggttatggaaggaatggcgattggaccacagggtt
        gggaagagaaactctagaggcagcctcttgaatttaggatttgcaagtcg  750
        atcccactttgacccctgaaagccattgacacggatggaggggaggagtg
        aaaactcatatgcaggcagctttctttaggccggggtgggacacaatgat
        agggtagtgagaagtctttggatccactagctagcagtatggccttggga
        aactccctctcatacctgggcttcttcctgaggctgcacccagggccctg
        gtttcacgatccagttctcactctaagcatcctcttggctcgcctgtgcc  1000
        cacagACCCCCTGCCAGCAGGAGTTGGACCAGGTCCTGGAACGGATCTCC
216     ThrProCysGlnGlnGluLeuAspGlnValLeuGluArgIleSer

        ACCATGCGCCTTCCGGATGAGCGGGGCCCCCTGGAGCACCTGTACTCCTT
231     ThrMetArgLeuProAspGluArgGlyProLeuGluHisLeuTyrSerLe

        GCACATCCCCAACTGTGACAAGCATGGCCTGTACAACCTCAAACAGgtga
248     uHisIleProAsnCysAspLysHisGlyLeuTyrAsnLeuLysGln

        gtgaggctctctggccgtgccttgctcccatcctacccctggaggtctca
        gagctgagccggagacctagtcttcatgagccaggggtggggatgtccgc  1250
        tgccaggccagggagagtgagggtggggcttgtttcttggcttcaggccc
        ctcggaggctgactccacttacctggctacctggcggcagcgctggtgtc
        caggatgggagggagccgcctggaaaagggatggggccttacaccaggg
        cgtcttgacttctgttgagcattaaaatcaccaggaggtgattcccaggc
        ctcacccccaaagactgccattcagggagttccccttcgcggctcagtggt
        aatgaccctgactag
```

Figure 3-3 -- continued

C. Exon 4

```
        cttagccctgcagaaccgcaagcacacccagacctgccgaagggcatctg  50
        catttcagtgcggcctcctccacctccacttctctctcctcagTGCAAGA
263                                                 CysLysM

        TGTCTCTGAATGGGCAGCGTGGGGAGTGCTGGTGCGTGAACCCCAACACC
266     etSerLeuAsnGlyGlnArgGlyGluCysTrpCysValAsnProAsnThr

        GGGAAGCTGATCCAGGGGGCCCCCACCATCCGGGGGGACCCCGAGTGTCA
282     GlyLysLeuIleGlnGlyAlaProThrIleArgGlyAspProGluCysHi

        TCTCTTCTACAACGAGCAGCAGGGGGCTCGCGGGGCACACACCCAGCGGA 250
299     sLeuPheTyrAsnGluGlnGlnGlyAlaArgGlyAlaHisThrGlnArgM

        TGCAGtaaaccacagccagccggtgcctggcacccccccccacccccaccc
316     etGln

        ctcttcaaacgcaggcagagcggggagagtgcttgcgtggtgggtggggg
        aggattttccaggagttctgacatgcgtatttatatttgaaagagacca
        gcaccgagcttggcacccgtccccccctccttcccccctccccagctgga
        gatgcccagctggagatgcccgcaccctccctcctggaatccctgctggg 500
        gagggaggggtggctgcagtggcggagctggggtagaggtttgggaggg
        gggaaaaaagaaatttttatttttgaaccccctgtgtctcttttgcttaag
        attaaaggaaggacaaataaagtgtgtgtcttttggctgaatcttttggg
        gtcccccctgggaaggagatcccgaggttcagggctcaccagcctcgtttt
        cttcagctttccccggaccctgggggaaatgggagaggcaagaatgggtt 750
        gtccactcaacaggaatgaaat
```

Table 3-1. The sequences of exon-intron boundaries and the 3' end of the IGFBP-2 gene.

		5' splicing site	3' splicing site
Consensus		AG/GTAAGT	Pyrmidine-rich NCAG/G
Intron I	Pig	AG/GTAACG	CTTGCTCTTTGCAG/A
	Human	AG/GTAACG	TCCTCTCTTGGCAG/A
	Rat	AG/GTAATG	TCTTCTCTCTGTAG/A
	Mouse	AG/GTAATG	GAGTCGACCTGCAG/A
Intron II	Pig	GG/GTCAGA	GCCTGTGCCCACAG/A
	Human	GG/GTCAGT	GTCTGTGCGTGCAG/A
	Rat	GG/GTCAGT	TTTGTCTCTTCAG/A
	Mouse	GG/GTCAGT	TTTTTTGTCTTCAG/A
Intron III	Pig	GG/GTCAGT	TTCTCTCTCCTCAG/T
	Human	GG/GTCAGC	TCCTCTCTCCCCAG/T
	Rat	GG/GTCAGT	CTCTTACCTTTCAG/T
	Mouse	GG/GTCAGT	TTTTTACCCTTCAG/T

3' end of the gene	
Pig	TAA...ATTAAAGGAAGGACAAATAAA(a)n
Human	TAA...ATTAAAGGAAGGAAAAGTA(A)n
Rat	TAA...ATTAAAGGAAGGACAAAT(A)n
Mouse	TAA...ATTAAAGGAAGGACAATAAAAGCTGCTGCTGTCTTACTTGAGTCTTTGGGG(A)n

```
P  MLPRLGGTAL SLLP..... ..LLLLLLG. .TGGRGARAE VLFRCPPCTP
H  MLPRVGCPAL PLPPPPLLPP LPLLLLLLGA SGGGGGARAE VLFRCPPCTP
B  MQPRLGGPAL LLLPPL.... ..LLLLLLGA GGGDCGARAE VLFRCPPCTP
D  MQPRLGGPAL LLLPPL.... ..LLLLLLGA GGGDCGARAE VLFRCPPCTP
R  MLPRLGGPAL PLLLPSL... ..LLLLLLGA GGCGPGVRAE VLFRCPPCTP
M  MLPR.GGPAL PLLLPSL... ..LLLLLLGA GGCGPGVRAE VLFRCPPCTP
                                                IGF Binding
P  ESLAACRPPP AAPPSAGAGP AGDSRAPC.E LVREPGCGCC SVCARLEGER
H  ERLAACGPPP VAPPAAVAAV AGGARMPCAE LVREPGCGCC SVCARLEGEA
B  ESLAACKP.. ..PPGAAAGP AGDARVPC.E LVREPGCGCC SVCARLEGER
D  ESLAACKP.. ..PPGAAAGP AGDARVPC.E LVREPGCGCC SVCARLEGER
R  ERLAACGPPP DA........ ......PCAE LVREPGCGCC SVCARQEGEA
M  ERLAACGPPP DA........ ......PCAE LVREPGCGCC SVCARQEGEA
      PKC                                  Tyr.
P  CGVYTPRCAQ GLRCYPHPGS ELPLQALVLG EGTCEKRRDA EYGASPEQVA
H  CGVYTPRCGQ GLRCYPHPGS ELPLQALVMG EGTCEKRRDA EYGASPEQVA
B  CGVYTPRCGQ GLRCYPNPGS ELPLRALVHG EGTCEKHGDA EYSASPEQVA
D  CGVYTPRCGQ GLRCYPNPGS ELPLRALVHG EGTCEKHGDA EYSASPEQVA
R  CGVYIPRCAQ TLRCYPNPGS ELPLKALVTG AGTCEKRR.. .VGATPQQVA
M  CGVYIPRCAQ TLRCYPNPGS ELPLKALVTG AGTCEKRR.. .VGTTPQQVA

P  DNGDD.AEGG LVENHVDGNV NLLGGTGGAG RKPLKSGMKE LAVFREKVTE
H  DNGDDHSEGG LVENHVDSTM NMLGGGGSAG RKPLKSGMKE LAVFREKVTE
B  DNGEEHAEGG LVENHVDGNV NLMGGGGGAG RKPLKSGMKE LAVFREKVTE
D  DNGEEHSEGG QVENHVDGNV NLMGGGGGAG RKPLKFRMKE LAVFREKVTE
R  DSEDDHSEGG LVENHVDGTM NMLGG.SSAG RKPPKSGMKE LAVFREKVNE
M  DSDDHHSEGG LVENHVDGTM NMLGGGSSAG RKPLKSGMKE LAVFREKVNE
                                                  PKC    _
P  QHRQMGKGGK MHLGLEEPKK LRPPPARTPC QQELDQVLER ISTMRLPDER
M  QHRQMGKGGK HHLGLEEPKK LRPPPARTPC QQELDQVLER ISTMRLPDER
B  QHRQMGKGGK HHLGLEEPKK LRPPPARTPC QQELDQVLER ISTMRLPDER
D  QHRQMGKGGK HHLGLEEPKK LRPPPARTPC QQELDQVLER ISTMRLPDER
R  QHRQMGKGAK .HLSLEEPKK LRPPPARTPC QQELDQVLER ISTMRLPDDR
M  QHRQMGKGAK .HLSLEEPKK LRPPPARTPC QQELDQVLER ISTMRLPDDR
   Tyr                                          PKC
P  GPLEHLYSLH IPNCDKHGLY NLKQCKMSLN GQRGECWCVN PNTGKLIQGA
M  GPLEHLYSLH IPNCDKHGLY NLKQCKMSLN GQRGECWCVN PNTGKLIQGA
B  GPLEHLYSLH IPNCDKHGLY NLKQCKMSLN GERGECWCVN PNTGKLIQGA
O  GPLEHLYSLH IPNCDKHGLY NLKQCKMSLN GQRGECWCVN PNTGKLIQGA
R  GPLEHLYSLH IPNCDKHGLY NLKQCKMSLN GQRGECWCVN PNTGKPIQGA
M  GPLEHLYSLH IPNCDKHGRY NLKQCKMSLN GQRGECWCVN PNTGKPIQGA
   PKC/RGD                 PKC
P  PTIRGDPECH LFYNEQQGAR GAHTQRMQ
M  PTIRGDPECH LFYNEQQEAR GVHTQRMQ
B  PTIRGDPECH LFYNEQQGAR GVHTQRMQ
O  PTIRGDPECH LFYNEQQGAR GVHTQRMQ
R  PTIRGDPECM LFYNEQQEND GVHAQRVQ
M  PTIRGDPECH LFYNEQQETG GAHAQSVQ
```

Figure 3-4. Deduced amino acid sequences of pig (P), human (H), rat (R) and mouse (M) IGFBP-2 proteins. Putative IGF binding site, protein kinase C phosphorylation sites (PKC PS), tyrosine phosphorylation sites (Tyr. PS) and Arg-Gly-Asp (RGD) integrin-binding sequence were identified by computer-assisted sequence analysis.

Table 3-2. Identity of porcine IGFBP-2 amino acid sequence with IGFBP-2 proteins of other species.

Exons	Human[a]	Bovine[b]	Ovine[c]	Rat[d]	Mouse[e]	Overall
E1	84.9	84.2	84.2	72.7	71.9	79.6
E2	90.8	94.7	89.5	81.6	81.6	87.6
E3	100	100	100	97.9	95.7	98.7
E4	96.3	96.3	98.2	87.0	87.0	93.0
Overall	90.8	91.1	90.2	81.0	80.0	

[a] Binkert et al., 1989
[b] Bourner et al., 1992
[c] Delhanty and Han, 1992
[d] Brown et al., 1989
[e] Landwehr et al., 1993

It was recently reported that the human IGFBP-5 gene is closely linked (distance of 20 to 40 kb) with the IGFBP-2 gene in a tail to tail orientation in chromosomal region 2q33-34 (Allander et al., 1994). Southern blot analysis using a human IGFBP-5 cDNA probe did not indicate hybridization to the pig cosmid 9-7, the insert of which contains exon 4 at one end and spans about 30 kb to the 3' side (data not shown). Therefore in the pig genome, the IGFBP-2 gene is separated from the IGFBP-5 gene by more than 30 kb.

Identification of 5'-Transcription Start Site(s)

In order to identify the transcriptional start site(s) of the pig IGFBP-2 gene, primer extension was initially employed. A 15 bp synthetic oligodeoxynucleotide primer was end-labeled and used in hybridization to poly(A)$^+$-RNA, total cellular RNA and yeast RNA (as negative control). Extension products from poly(A)$^+$ and total cellular RNA but not from yeast RNA were observed with 5'-ends at positions corresponding to -96 to -87 (relative to the ATG initiation codon), suggesting multiple clustered initiation sites (Figure 3-5A). However, these signals were relatively weak and unresolved. Therefore, S1-nuclease protection was used to corroborate or refute the results from primer extension. Again, multiple bands were observed that indicated heterogeneous 5' termini at positions -96 to -87 (Figure 3-5B). Interestingly, when the Hela cell *in vitro* transcription system and an RNase protection assay (RPA) were used to further confirm the locations of these transcription start sites, strong signals were found (especially in the RPA) not only at positions -96 to -87 but also slightly upstream at positions -109 to -105 (Figure 3-5C and D). Overall, the results from these four independent experimental approaches indicated that the TATA box-less and GC-rich promoter region of the pig IGFBP-2 gene exhibits two clusters of transcriptional

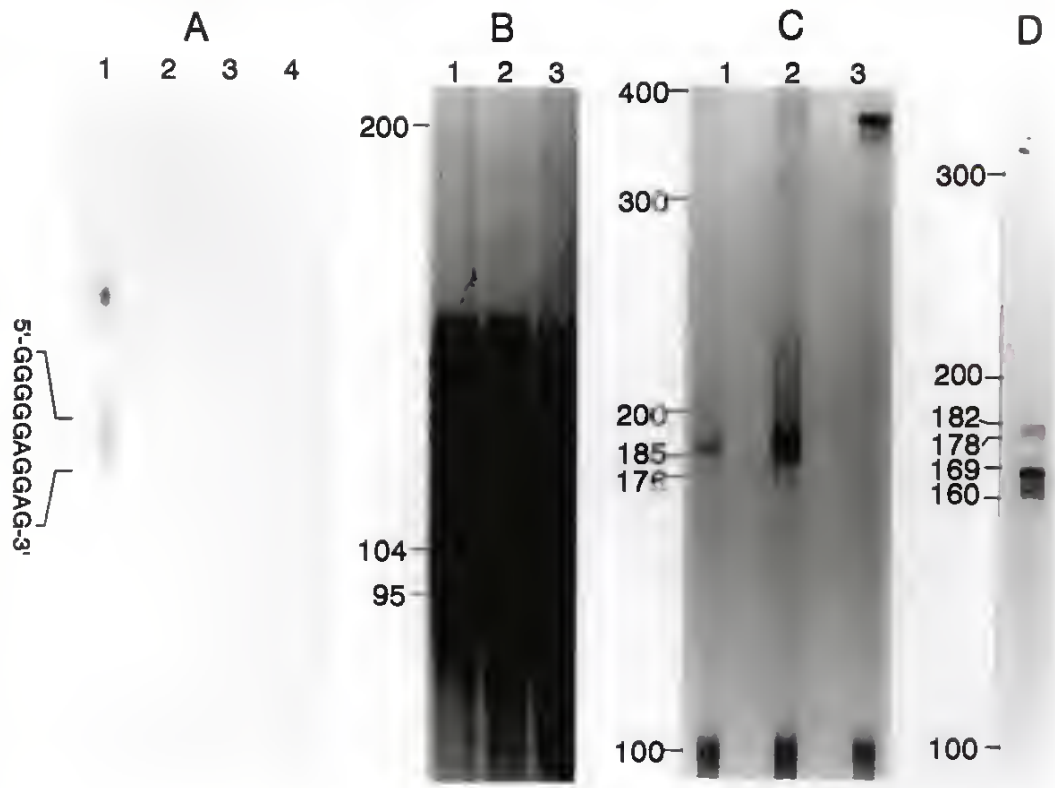


Figure 3-5. Identification of 5'-transcriptional start sites. Panel A. Results of primer extension assay. Primers were annealed to 2 µg of poly $(A)^+$ RNA (lane 1) or, 50 µg of total cellular RNA (lane 2) from endometrium of a Day 60 pregnant pig, 50 µg of yeast tRNA as control (lane 3) or no RNA (lane 4), respectively. Sequencing reactions using the same primer were co-electrophoresed and the sequence corresponding to the extended products is shown on the left. Panel B. Results of S1 nuclease protection assay. The ^{32}P-labeled probe was hybridized to 10 µg of total cellular RNA from endometrium of Day 30 (lane 1), Day 60 (lane 2) and Day 90 (lane 3) pregnant pigs. DNA size markers (100 bp ladder, Gibco BRL, Life Technologies, Inc., Grand island, NY) were run on the same gel and their migration positions are indicated on the left. Panel C. Results of Hela cell *in vitro* transcription assay. Templates used for the *in vitro* transcriptions were 100 ng (lane 1) or 200 ng (lane 2) of *Sma*I + *Hin*dIII fragment, or 100 ng of CMV immediate early promoter DNA (lane 3, as positive control). RNA size markers transcribed by T7 RNA polymerase using Century Marker Templates (Ambion, Inc., Austin, Texas) were run on the same gel and their migration positions are indicated on left. Panel D. Results of RNase protection assay. Five ug of total cellular RNA from the endometrium of a Day 60 pregnant pig was hybridized to the ^{32}P-labeled probe. The positions of the RNA markers are indicated on the left.

start sites (at -109 to -105 and -96 to -87) of which the cluster at -96 to -87 appears to be the predominantly utilized one (Figure 3-6).

Insights from Analysis of the Upstream Region of the IGFBP-2 Gene

Comparisons of the sequences of the porcine, human, rat and mouse IGFBP-2 gene 5' flanking regions (to the ATG translation initiation codon) were performed using the currently available sequences. The highest overall homology was observed for the DNA region spanning the G/C boxes to the ATG codon (Fig 3-7). Smaller blocks of conserved sequence were apparent in the more upstream region, several of which overlapped with transcription factor binding site motifs: e.g., a DBP site (Faisst and Meyer, 1992), a nonconsensus estrogen-response element (ERE) and two ERE half sites (Dana et al., 1994) (Figure 3-7). Two non-conserved progesterone-response element (PRE) half sites (Lieberman et al., 1993) were also identified in the 5' flanking region of the pig IGFBP-2 gene.

Discussion

This study has extended previous investigations of the pig uterine IGF/IGFBP system during pregnancy. A close temporal association of IGFBP-2 mRNA and maximal circulating estrogens and an inverse relationship of IGFBP-2 mRNA with circulating progesterone in sexually mature pigs was noted previously (Simmen et al., 1992). However, estrogen or the combination of estrogen and progesterone were ineffective in causing an induction (or repression) in uterine IGFBP-2 mRNA level in prepubertal pigs (Simmen et al., 1990). Results of the present study indicated maximal IGFBP-2 mRNA abundance in pig endometrium during the mid-pregnancy. This pregnancy-stage specific gene expression seems not simply correlated with neither estrogen nor progesterone levels in maternal serum.

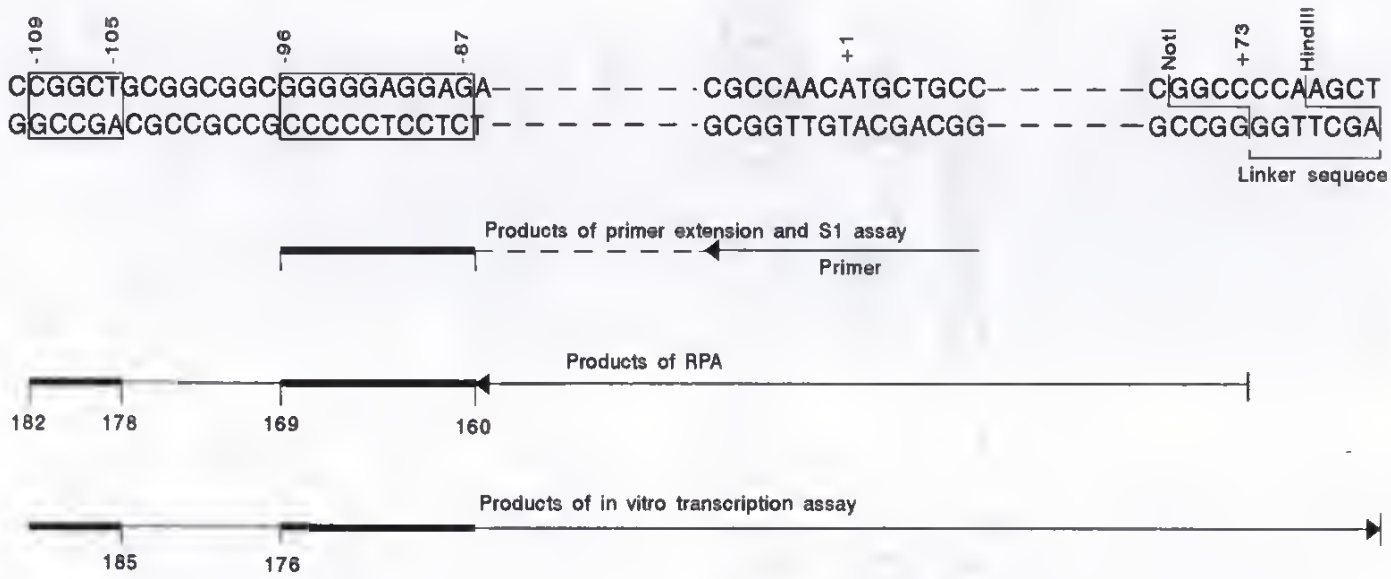


Figure 3-6. Summary of the results of the localization of the 5'-ends of IGFBP-2 mRNAs. Clusters of transcriptional start sites are boxed.

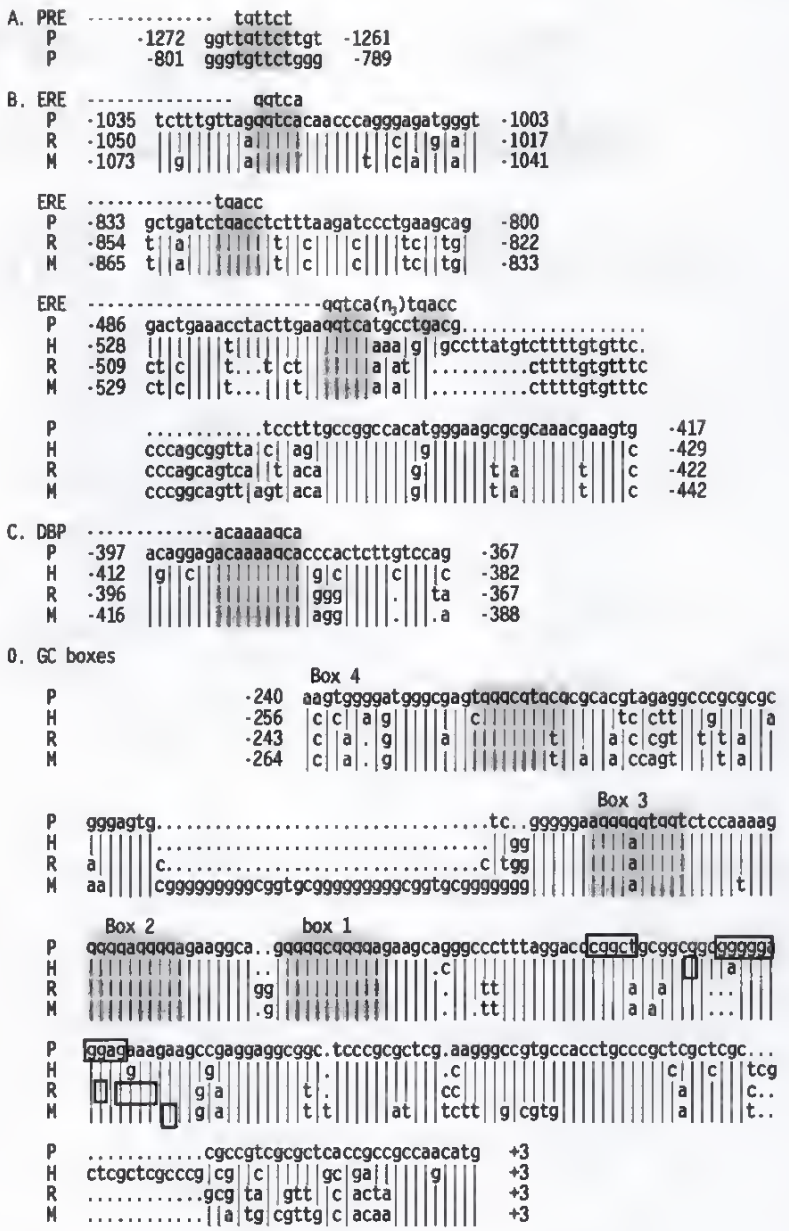


Figure 3-7. Conserved sequence motifs in the region upstream of the translational initiation codon of the IGFBP-2 gene. Corresponding sequences of the porcine (P), human (H) (Binkert et al., 1992), rat (R) (Brown and Rechler, 1990) and mouse (M) (Landwehr et al., 1993) IGFBP-2 genes were aligned by use of the Group and Pileup programs of the GCG Package. Two PRE half sites in the porcine sequence are shown in **A**. Localized regions of homology which overlap ERE half sites and imperfect palindrome (**B**), DBP site and G/C boxes (**D**) are shaded. The transcription start sites are boxed.

In human endometrium, IGFBP-2 and IGFBP-3 mRNA abundance and protein synthesis and secretion are greater in the secretory than proliferative phase of the estrous cycle, suggesting a positive association with increased serum progesterone (Giudice et al., I991a, b). Moreover, cultured endometrial stromal cells from women exhibit enhanced IGFBP-2 and IGFBP-3 secretion and correspondingly increased mRNA levels at ~6 days after administration of estrogen and/or progesterone (Giudice et al., I991a). The effects of steroid hormones on uterine production of IGFBPs may be immediate (i.e., steroid receptor transactivation of IGFBP promoter), indirect, perhaps via the induction or repression of intermediate proteins such as endometrial-specific transcription factors or a combination of both mechanisms.

Results from this and earlier studies (Simmen et al., I990, I992; Geisert et al., I99I) identified marked inductions in endometrial IGFBP-2 mRNA steady-state levels beginning just after initiation of implantation. It is interesting that pig endometrial IGFBP-2 transcripts and epithelial IGFBP-2 are maximally expressed after day 30, the time at which the porcine placenta becomes completely formed (King, I993). The pig exhibits diffuse epitheliochorial placentation with no invasiveness of the trophoblast. Therefore, IGFBP-2 may be an important mediator of endometrial/placental interactions that underlie degree of trophoblast invasiveness, placental development and/or nutrient transfer across the placenta.

IGFBP-2 has been localized on membranes of cells that synthesize this protein (Reeve et al., I993; Chapter 5) where it competes for binding of IGFs to receptors. A negative correlation of endometrial IGF-I and IGFBP-2 mRNA abundance was recently observed (Song et al. I996) and may indicate that IGF-I is an inhibitor of endometrial

IGFBP-2 gene expression. One interesting discrepancy relates to the absence of immunostaining for IGFBP-2 in endometrial stroma at mid-pregnancy (Song et al., 1996). These cells have been shown to express the IGFBP-2 gene (Simmen et al., 1992). This may indicate either a block in translation of the stromal IGFBP-2 mRNA or the rapid transfer of IGFBP-2 from stromal cells to endometrial epithelial surfaces during its biosynthesis and secretion.

The coding and deduced amino acid sequences, predicted exon-intron junctions and polyadenylation site for pig IGFBP-2 reported here are in complete agreement with the corresponding sequences of an unpublished, full-length porcine IGFBP-2 cDNA cloned from vascular smooth muscle cells in the laboratory of D.R. Clemmons (personal commun., D.R. Clemmons). In addition, Coleman et al. (1991) reported a partial amino-terminal amino acid sequence for porcine serum IGFBP-2. Their sequence initiated 3 amino acid residues downstream of the glutamic acid residue considered to be the first residue of mature IGFBP-2 (Brown et al., 1989; Bourner et al., 1992; Drop et al., 1992) and was in agreement with that elucidated here by sequencing of the first exon of the pig IGFBP-2 gene. The porcine IGFBP-2 protein exhibits the conserved pattern of 18 cysteine residues clustered at both termini of the protein as well as the Arg-Gly-Asp (RGD) motif in its carboxyl-terminus. Putative protein kinase C and tyrosine kinase phosphorylation sites were found in this protein by computer analysis. However, the functions of these sites are yet to be tested. The overall sequence conservation of IGFBP-2 molecules of different mammalian species is very high (80-91%).

The promoter of the IGFBP-2 gene is TATA-less and GC-rich. Computer assisted sequence analysis indicated that the corresponding mRNAs can form hair-pin secondary structures (data not shown). In this study, primer-extension analysis yielded a very weak signal due to the low efficiency of reverse transcription possibly caused by mRNA secondary structures. Although the DNA sequences of the IGFBP-2 gene promoter region are highly conserved in the pig (this study), the human (Binkert et al., 1992), the rat (Brown and Rechler, 1990; Kutoh et al. 1993; Boisclair and Brown,1995) and the mouse (Landwehr et al., 1993) (Figure 3-6), the transcriptional start sites are not the same. TATA and CAAT-less promoters generally have multiple transcriptional start sites (for reviews see McKeon et al., 1990; Lu et al., 1994). In the present study, two clusters of transcriptional start sites were identified. The major cluster at -96 to -87 is located at a similar position as the transcription start site(s) of the human, rat and mouse IGFBP-2 genes. However, the minor cluster at-109 to -105 may represent initiation sites unique to the pig IGFBP-2 gene. Although this cluster of start sites was not clearly demonstrated by primer extension and S1-nuclease protection assays, *in vitro* transcription and RNase protection assays clearly demonstrated its occurrence.

The IGFBP-2 exon-1 region and 5' flank is characterized by a number of G/C boxes (GGGCGG or the reverse complement). These motifs can constitute cis-acting elements for a number of different DNA-binding proteins such as Sp1 and related factors and were previously shown to be essential for basal transcriptional activity of the rat IGFBP-2 gene (Boisclair et al., 1993; Kutoh et al., 1993). A number of G/C box-binding proteins, including SP1, SP3 and BTEB are present in porcine uterine endometrium of pregnancy (Wang, Y. and

Simmen, R.C.M., unpublished results). Sp1 binding to *cis* elements may also serve as part of a mechanism to protect CpG islands from *de novo* methylation (Brandeis et al., 1994; Macleod et al., 1994). For tissue-specific genes, a strong correlation exists between expression and undermethylation (Razin and Cedar, 1991). The location of this CpG island (bracketing the transcriptional initiation site, translational start site, leader peptide and remainder of exon 1), its strong evolutionary conservation, and potential for differential methylation point to its probable function in transcriptional and perhaps, post-transcriptional regulation of expression of this gene *in utero*.

Summary

In this study, endometrial-specific and temporally regulated expression of the pig uterine IGFBP-2 gene was confirmed. In particular, maximal expression of the IGFBP-2 gene was found around mid-pregnancy. As a prerequisite to understanding the molecular mechanisms of this gene's regulation, the genomic structure of the IGFBP-2 gene, which is comprised of four exons spanning ~29 kb and which encodes a 316 amino acid precursor protein, was elucidated. The TATA and CAAT box-less promoter region of this gene exhibits two clusters of transcriptional start sites located -109 and -87 relative to the translational initiation codon. Lastly, putative transcription factor binding sites in the upstream 1.4 kb region flanking the IGFBP-2 gene were identified. Several of these motifs may be associated with molecular mechanisms by which circulating steroid hormones affect endometrial IGFBP-2 mRNA and protein steady-state levels during the estrous cycle and pregnancy.

CHAPTER 4
IDENTIFICATION OF *CIS*-ELEMENTS IN THE UPSTREAM ACTIVATING REGION OF THE IGFBP-2 GENE

Introduction

IGF-I and -II undoubtedly play important regulatory roles in feto-maternal interactions (Simmen et al., 1995) as in many other physiological processes. IGF binding proteins (IGFBPs), a family of at least six members, are modulators of IGF actions in the circulation and at the cellular surface (Jones and Clemmons, 1995). Functions and expression of each these IGF binding proteins exhibit tissue-specificity, developmental-specificity and hormonal regulation (Rechler and Brown, 1992; Clemmons, 1993). In the pig uterus, the IGFBP-2 gene has a relatively unique pattern of mRNA expression during pregnancy. The expression of this gene becomes induced at periimplantation to reach maximal levels at mid-pregnancy. Unlike the other uterine-expressed IGFBPs such as IGFBP-3, -4 and -5 and other uterine genes such as uteroferrin and IGF-I (Simmen et al., 1992; Song et al., 1996), the IGFBP-2 gene is highly expressed in uterine endometrium, with little or no mRNA expressed in the corresponding myometrium during pregnancy. Therefore, this gene may constitute a useful model system to study endometrial-specific and pregnancy-dependent modes of transcriptional and post-transcriptonal regulation as well as identify novel uterine endometrial transcription factors.

The genomic organization of the IGFBP-2 locus has been characterized in human (Ehrenborg et al., 1991; Binkert et al., 1992), rat (Brown and Rechler, 1990; Kutoh et al., 1993), murine (Landwehr et al., 1993) and chicken (Schoen et al., 1995). Unlike the other IGFBPs, the IGFBP-2 gene has a TATA box-less promoter, which is highly GC-rich and conserved across species (see Figure 3-7.). There are several GC boxes in the conserved promoter region. In rat, it has been shown that four GC boxes are required for binding of transcription factor SP1 to elicit basal transcription. Moreover, the further upstream or distal region also contains some conserved sequences and putative transcription factor binding sites such as half EREs, half PREs, DBP and AP1 sites. In pregnant pig endometrium, SP1 expression is constitutively low and is not correlated to the pattern of expression of IGFBP-2 RNA during pregnancy (Y. Wang and R.C.M. Simmen, unpublished data). Although this GC rich gene promoter may interact with SP1 or other GC box binding proteins in uterine endometrium, the distal upstream region may also be involved in the regulation of this gene. However, the potential effects of this distal region on transcriptional activity of the IGFBP-2 gene have not been previously studied in any species.

In the previous chapter, the structure of the porcine IGFBP-2 gene was characterized. This gene was shown to contain four exons and three introns spanning ~29 kb and encoding for a 316 amino acid IGFBP-2 precursor protein. Two clusters of transcriptional start sites were identified at about 100 bp upstream of the ATG initiation codon in exon 1 and approximately 1.4 kb of the 5'-flanking region was sequenced. The present study has extended these structural findings by focussing on the identification of functional upstream

region(s) and corresponding *cis*- and trans-elements within this region that may confer, in part, the high level endometrial expression of the IGFBP-2 gene during pregnancy.

Materials and Methods

Reagents and Enzymes

All reagents used were of molecular biology grade. Oligonucleotides were synthesized by the DNA Synthesis Core Laboratory of the Interdisciplinary Center for Biotechnology Research (ICBR) at the University of Florida. Restriction enzymes and poly(dI-dC) were purchased from Boehringer-Mannheim Biochemicals (Indianapolis, IN); Radionucleotides were purchased from ICN Pharmaceuticals, Inc. (Irvine, CA); RPMI 1640 medium, Hanks' Balanced Salt Solution and Pancreatin were purchased from Gibco BRL, Life Technologies, Inc. (Grand island, NY); DME/F-12 (1:1 mixture), Calf thymus DNA, fetal bovine serum (FBS) and ABAM (Antibiotic-Antimycotic) were purchased from Sigma Chemical Co. (St. Louis, MO).

Plasmid Construction

The plasmid H6 (see Chapter 3) was cleaved with *Not*I (at nucleotide position +73 relative to the translational initiation codon). The adhesive ends were made blunt-ended by treatment with the Klenow fragment of DNA polymerase I and ligated to a synthetic *Hin*dIII linker (Promega, Corp., Madison, WI) and the DNA cleaved with *Hin*dIII. A fragment, spanning from -1389 to +73 relative to the ATG, and also containing 3 bp of linker sequence on the 3'-end, was isolated from an agarose gel and cloned in the pGL2-enhancer vector (Promega, Corp., Madison, WI) at the *Hin*dIII site to construct the plasmid Hind-LUCe. Hind-LUCe was cleaved with *Sac*I, *Bgl*II or *Sma*I. The large fragments containing the

upstream regions from -874, -764, or -305 to +73, respectively and the linked vectors were isolated from agarose gels and religated to create Sac-LUCe, Bgl-LUCe, and Sma-LUCe, respectively. Sac-LUCe was cleaved with *Sac*I and *Bgl*II, a fragment of 110 bp (spanning from -874 to -765) was isolated from an agarose gel using DEAE paper, and inserted in the sense orientation to the pGL2-promoter vector (Promega, Corp., Madison, WI) at the unique *Sac*I and *Bgl*II sites to create SaB (110)-pLUC. Bgl-LUCe was cleaved with *Bgl*II and *Sma*I, a fragment of 459 bp spanning from -764 to -306 was isolated from an agarose gel using DEAE paper, and this was inserted in an antisense orientation in pGL2-promoter vector to construct SmB (459)-pLUC. PCR products from -874 to -765, generated with a 5'-primer (SB5) 5'-CCAAAGGACCTAACCTCA-3' and a 3'-primer (SB3) 5'-TCCAGACATTTGCT GCAG-3', were treated with S1 nuclease and inserted into the pGL2-promoter vector at the unique *Sma*I site. Ten clones were subjected to sequence analysis and two of these were found to have inserts. One contained an insert of 42 bp fragment from -829 to -788 in the antisense orientation and was named 42(+)Sma-LUCe. The other contained an insert of 25 bp from -812 to-788 in the sense orientation, and was named 25(-)Sma-LUCe. All DNA constructs were sequenced to confirm orientation and identity of chimeric inserts. Plasmid DNAs used for transfection were prepared using a DNA purification kit (Qaigen Inc. Chatsworth, CA) or by the cesium chloride method (Sambrook et al., 1989).

Propagation of Primary Cell Cultures and Cell Lines

Gilts were monitored twice daily for onset of estrous activity. Gilts exhibiting two consecutive estrous cycles of normal duration (18-22 days) were mated at estrus with boars and again 12 and 24 h later. The day of onset of estrus was defined as Day 0 of pregnancy.

Animals were sacrificed at the University abattoir on the indicated days of pregnancy. Reproductive tracts were removed, immersed in ice and trimmed from the mesometrium. Endometria, myometria and placentae were obtained by dissection as previously described (Simmen et al., 1990b; Ko et al., 1994a). Animal use protocols were approved by the University of Florida Institutional Animal Care and Use Committee.

Primary cultures of uterine cells were obtained as described previously (Zhang et al., 1994; Reed et al. 1996). Briefly, the uterus was removed and flushed with Phosphate-Buffered Saline (PBS) containing 1% ABAM (Antibiotic Antimycotic: 500 U/ml penicillin, 500 μg/ml streptomycin, 25 μg/ml amphotericin B). Endometrium was obtained by dissection and washed three times with Hank's Balanced Salt Solution (HBSS) containing 1% ABAM and 50 μg/ml gentamicin. The tissue was incubated in digestion solution I (4.8 mg/ml dispase and 12.5 mg/ml pancreatin in HBSS) for 2.5 hours at room temperature. The tissue was then washed six times in HBSS containing 1% ABAM to obtain luminal epithelial cells. The residual tissue was incubated in digestion solution II (0.04% trypsin, 0.06% collagenase, 0.01% DNase I in HBSS) for 50 minutes at 37 °C to dissociate into a single cell suspension. The cell suspension was separated into glandular epithelial and stromal cells by passage through a 38 μm stainless steel sieve and separated cells were seeded in 35 mm well, 6-well plates in RPMI 1640 medium containing 10% FBS and 1% ABAM. Medium was changed every 2 to 3 days until cultures were ready to be transfected.

The human endometrial cell line ECC-1 was propagated in RPMI 1640 medium containing 10 % FBS, 1 % ABAM, 25 μg/ml transferrin, 6 mM glutamine, 10 ng/ml cholera toxin, 0.11 % Na bicarbonate, 20 mM Hepes, and 200 mg/L glucose. The human

trophoblastic choriocarcinoma cell line JEG-3 was propagated in DME/F-12 medium containing 1 % ABAM and 10 % FBS.

Transfection of Primary Cell Cultures and Cell Lines

Cells were detached from plates by treatment with trypsin and collected by centrifugation at 2000 rpm for 3 minutes. The cell pellet was resuspended in transfection buffer [Hepes Buffered Solution (HBS): 21 mM Hepes, pH 7.05, 137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, 6 mM Glucose)]. Cells were transfected with luciferase reporter plasmids (20 μg) by electroporation (Cell-Porator, GIBCO BRL, Life Technologies, Inc., setting at 200-250 voltage and 330 μF) at room temperature. The transfection efficiency was normalized for total protein concentration in cell extracts or for pCAT activity which was determined by co-transfection of pCAT plasmid (10 μg)(Promega, Corp., Madison, WI) with the Luciferase reporter plasmids. Forty-eight hours after transfection, cells were washed twice with PBS, and lysed in 200 μl of cell lysis buffer (Promega, Corp., Madison, WI). One hundred μl of cell lysate was saved for protein or CAT assay. Another 100 μl was centrifuged at 12000 rpm for 5 min. Thirty μl of supernatant was used to determine luciferase activity with luciferase substrate (Promega Corp., Madison, WI) for 3 min in a luminometer (AutoLumat, LB953, EG&G Berthold). Protein concentrations were determined by the Bradford method using bovine serum albumin as standard. For CAT assays, 100 μl of lysate was incubated at 65 °C for 10 min and centrifuged at 12000 rpm for 10 min. Eighty μl of supernatant was adjusted to a volume of 125 μl of 0.25 mM Tris-HCl, pH 8.0, 0.08 mCi ^{14}C chloramphenicol and 25 μg of n-butyryl CoA, and incubated at 37 °C overnight. After one

extraction with mixed xylenes and one back extraction with 0.25 mM Tris-HCl, 200 µl of xylenes containing the radiolabeled CoA was counted in a liquid scintillation counter.

Transfection data were analyzed by least squares analysis of variance using the General Linear Models procedure of the Statistical Analysis System (SAS; Barr et al. 1979). In the model, the effects of experiment or pig (as random effect), constructs and their interactions on luciferase activities were considered. Protein concentration and/or pCAT activity were used as covariates to normalize the luciferase activity. Differences between constructs were determined using orthogonal contrasts.

Preparation and Fractionation of Nuclear Extracts

Nuclear extracts were prepared as described previously (Gonzalez et al. 1994) with some minor modifications. Briefly, 2 g of frozen tissue was minced in a Waring Blender using 20 ml of homogenization buffer (10 mM Hepes, pH 7.6, 25 mM KCl, 1 mM EDTA, 1.5 M sucrose, 10% glycerol) supplemented with 0.15 mM spermine, 0.5 mM spermidine, 4 ug/ml aprotinin, 0.1 mM phenylmethlsulfonyl fluoride (PMSF) and 0.2 mg/ml trypsin inhibitor. The homogenate was transferred to a Dounce homogenizer on ice and further disrupted by three strokes. Nuclei were centrifuged twice through 10-ml sucrose cushions (30,000 X g for 30 min). Nuclear pellets were resuspended in a 6 X volume of nuclear lysis buffer [10 mM Hepes, pH 7.6, 100 mM KCl, 3 mM $MgCl_2$, 0.1 mM EDTA, 1 mM dithiothreitol (DTT), 0.1 mM phenylmethlsulfonyl fluoride (PMSF), 10% glycerol], and 4 M ammonium sulfate was added dropwise with stirring to a final concentration of 0.3 M, followed by gentle mixing for 30 min at 4 °C using a rocking platform. Extracts were centrifuged at 150,000 X g, for 1 hour at 4 °C. Ammonium sulphate (0.3 g/ml of extract)

was then added to the supernatant with gentle stirring. The nuclear protein precipitate was pelleted by centrifugation at 50,000 X g for 15 min and was dialyzed against buffer (25 mM Hepes pH 7.6, 40 mM KCL, 0.1 mM EDTA, 1 mM DTT, 10% glycerol) overnight. Protein concentrations were determined by the Bradford method using bovine serum albumin as standard. Nuclear extracts were subdivided into 25 ug aliquots and stored at -80 °C until use.

Fractionation of nuclear extracts followed the procedure described by Gonzalez et al. (1994). Nuclear extracts were loaded on a pre-equilibrated (100 mM NaCl in dialysis buffer) DEAE BioGel A column (Bio-Rad, Richmond, CA) at 4 °C and eluted with a linear salt gradient (0.1 to 1.0 M NaCl) in dialysis buffer. Fractions were collected and the protein concentration of each fraction was measured. The flow-through fractions were pooled and designated as Fraction 1.The eluted fractions were separated and pooled as Fractions 2 to 6.

Gel Retardation Assay (GRA)

The procedure used for the gel retardation assay was modified slightly from that described by Gonzalez et al. (1994). Briefly, 5 to 20 μg of crude or fractionated nuclear proteins were preincubated in binding buffer (10 mM Tris-HCl, pH 7.4, 60 mM KCl, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM DTT, and 10% glycerol) also containing 4 ug of sonicated calf thymus DNA and 2 μg of poly(dI-dC) with or without unlabeled competitor for 15 min on ice. DNA probes [^{32}P-labeled (1.0-1.5 X 10^5 cpm) restriction enzyme-generated DNA fragments or synthetic oligonucleotides] were added to the binding mixture and incubated on ice for an additional 30 min. Binding complexes were monitored in low-ionic strength, 4 or 6 % polyacrylamide gels (acrylamide/bis-acrylamide = 29:1) with TAE buffer (10 mM

Tris-HCl, pH 7.4, 50 mM HoAc, 1 mM EDTA) and electrophoresed at room temperature. Gels were dried and exposed to X-ray film for overnight to 4 days.

All double-stranded oligonucleotides, except for probes A and C, were generated by annealing complementary oligodeoxynucleotides in annealing buffer (40 mM Tris-HCl, pH 7.5, 20 mM $MgCl_2$, 50 mM NaCl) first by heating at 65 °C for 20 min, and then incubating at 37 °C for 20 min followed by placement at room temperature for 20 min. Probe A was generated by annealing of an 18 nt primer (SB5) and a 40 bp oligonucleotide, of which the 3'-end was the complement of the SB5 primer. The hybrid was made double-stranded using the Klenow fragment of DNA polymerase. Similarly, Probe C was generated from the SB3 primer and a 40 nt oligonucleotide, of which the 3'-end was the complement of SB3.

UV Cross-Linking

Standard procedures for UV cross-linking as previously described by Williams and Konigsberg (1991) were used. The ^{32}P-end labeled A2 probe was incubated with nuclear extracts in the same buffer used for GRA by placement on ice for 30 min. The final reaction volume was 15 ul. The binding reactions were exposed to UV light (254 nm, using a hand-held short wave UV light box, 115 V, 60 Hz, 0.16 A) at a distance of 10 cm for 5 min. The cross-linked products were examined by SDS-PAGE. Gels were dried and exposed to X-ray film (autoradiography).

Southwestern Blot

A protocol modified from that described by Harrison et al. (1991) was used. A total of 50 to 200 ug nuclear protein or fractions of nuclear extracts were treated with β-

mercaptoethanol and subjected to SDS-PAGE using a Bio-Rad mini gel apparatus. The proteins in the gels were electrotransfered to nitrocellulose filters. The membranes were incubated in TEN buffer (10 mM Tris-HCl pH, 7.4, 1 mM EDTA, 60 mM NaCl) containing 5 % Carnation non-fat dry milk by gently rocking at room temperature for 20 min. After blocking, the membrane was washed twice with TEN and then incubated in TEN containing sonicated calf thymus DNA (5 μg/ml) and the presence or absence of unlabeled competitor DNA for 15 min. Then, radiolabeled probe (A2; 7 X 10^6 cpm/ml) was added, and incubation at 4 °C continued for 24 hours. After two 5 min washes in TEN, the membrane was exposed to X-ray film.

Results

Identification of Functional Upstream Regulatory Regions of the IGFBP-2 Gene

In cancer cell lines. As an initial step for the identification of functional upstream regulatory regions, the promoter constructs Hind-LUCe (-1397/+73), Sac-LUCe (-874/+73), Bgl-LUCe (-764/+73) and Sma-LUCe (-305/+73) (Figure 4-1) were separately transfected into the human endometrial carcinoma cell line ECC-1 (known to express the endogenous IGFBP-2 gene) and the human trophoblastic cell line JEG-3 (Figure 4-2). Data from these transient transfections showed that deletion from -874 to -765 decreased luciferase activity (2 to 2.5 fold), while deletion from -764 to -306 increased luciferase activity in both cell lines. However, in Ecc-1 but not JEG-3 cells, deletion from -1397 to -875 increased reporter gene activity. These data therefore indicate that the region from -874 to -765 may have positive effects on promoter activity, whereas regions from -1397 to -875 (in ECC-1), and from -764 to -306 (in both cell types), may have transcriptional inhibitory effects.

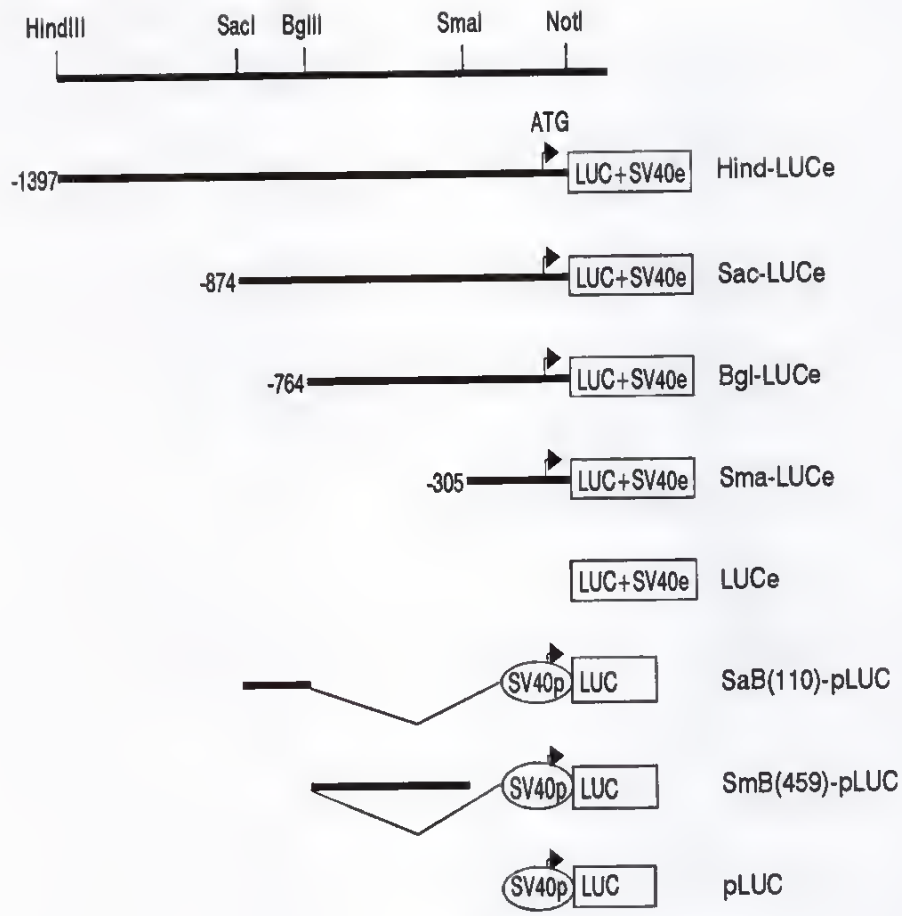


Figure 4-1. DNA constructs containing the upstream region of the IGFBP-2 gene fused to the luciferase reporter gene. The top line presents the restriction endonuclease map of the upstream region of the pig IGFBP-2 gene. The number for each construct designates the 5' end of the fragment relative to the translational initiation codon (ATG) of IGFBP-2 (+1). The open boxes represent the luciferase coding sequence (LUC). The reporter genes (pGL2-enhancer) with SV40 enhancer (SV40e) and without SV40 promoter are labeled as LUCe; the reporter genes (pGL2-promoter) with SV40 promoter and without enhancer are labeled pLUC. Hind, Sac, Bgl, and Sma represent cleavage sites for *Hin*dIII, *Sac*I, *Bgl*II and *Sma*I respectively.

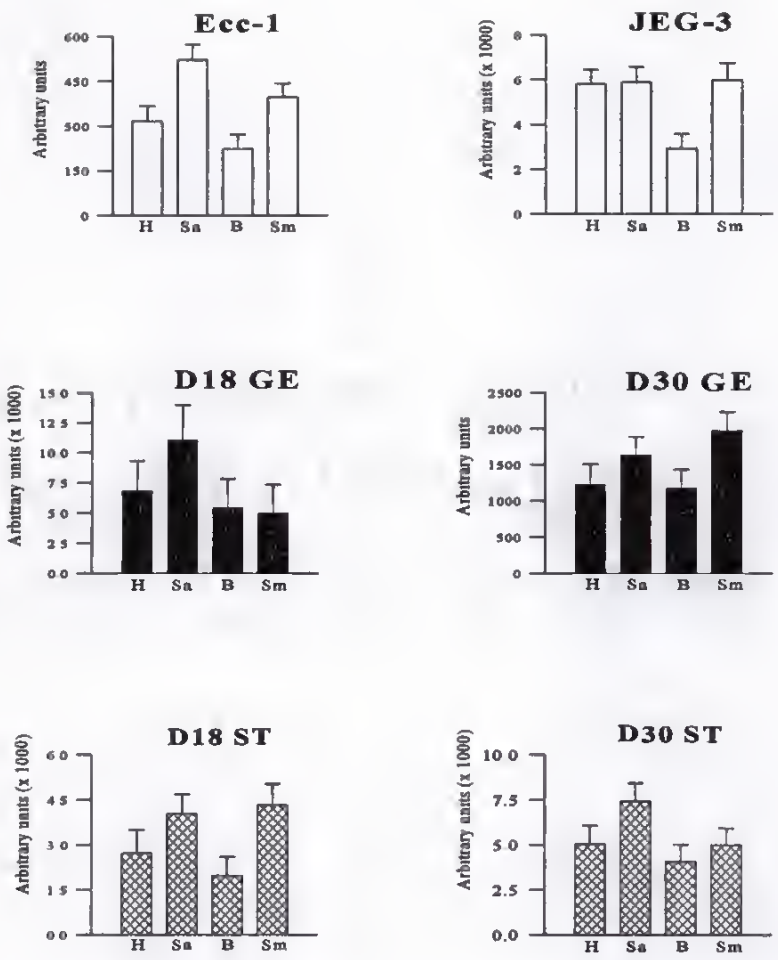


Figure 4-2. Reporter gene activities of the Hind-LUCe, Sac-LUCe, Bgl-LUCe and Sma-LUCe plasmids transfected in different cell lines and uterine cell types. Each bar represents the Least Squares Mean (n=9) of triplicate data in arbitrary light units (normalized for protein concentration of cell extract) from three experiments (n=3 pigs). Hind-LUCe, Sac-LUCe, Bgl-LUCe and Sma-LUCe are labeled as H, Sa, B and Sm, respectively. The open bars represent the data from transfected cancer cell lines. In ECC-1 cells, Sa is significantly higher than H ($P<0.05$) and B ($P<0.01$), and Sm is significantly higher than B ($P<0.05$). In JEG-3 cells, B is significantly lower than both Sa ($P<0.05$) and Sm ($P<0.05$). The black bars represent the data from transfected uterine endometrial glandular epithelial cells of pigs. In transfected glandular epithelial cells from the endometrium of Days 18 (D18 GE) and Day 30 (D30 GE) pregnant pigs, no statistically significant difference was found between constructs. The stippled bars represent the data from transfected uterine endometrial stromal cells. In stromal cells from endometria of Days 18 (D18 ST) pregnant pigs, Sm is significantly higher than B ($P<0.05$). In stromal cells from endometrium of Day 30 (D30 ST) pregnant pigs, Sa is significantly higher than B ($P<0.05$).

In primary cultures of uterine endometrial cells. Continuous porcine endometrial cell lines are not currently available. Therefore, the above constructs were transfected into primary cultures of uterine endometrial glandular epithelial (GE) and endometrial stromal (ST) cells isolated from endometrium of Day 18 and Day 30 pregnant pigs. Results from DNA transfections of the two uterine cell types representing the two stages of pregnancy are shown in Figure 4-2. Similar to the results from, deletion of sequence from -874 to -765 decreased luciferase activity, whereas deletion from -1397 to -875 increased luciferase activity. However, effects of deletion from -764 to -306 were observed differentially in transfected D18 GE, D30 GE, D18 ST and D30 ST cells. GE cells from Day 18 pregnant pigs yielded the highest overall IGFBP-2 promoter activity. Therefore, D18 GE cells were utilized for subsequent experiments.

Although the above data for the pig endometrial cells suggested that the 110 bp region from -874 to -765 may have an inducing or activating function, the relatively large variation in the data may have diminished the overall statistical significance of this effect. Therefore, the constructs Sac-LUCe, Bgl-LUCe and LUCe (promoterless control) were cotransfected with pCAT into GE cells from Day 18 pregnant pigs (to correct for variability of transfection efficiency). The normalized data clearly showed that deletion from -874 to -765 decreased the reporter gene activity by 75%, again suggesting that this region is stimulatory for IGFBP-2 promoter activity in these cells (Figure 4-3).

Enhancing Activity of the 110 bp Region Extending from -874 to -765

To examine the activity of the 110 bp region within the context of a heterologous promoter, the construct SaB(110)-pLUC was generated by inserting the DNA fragment from

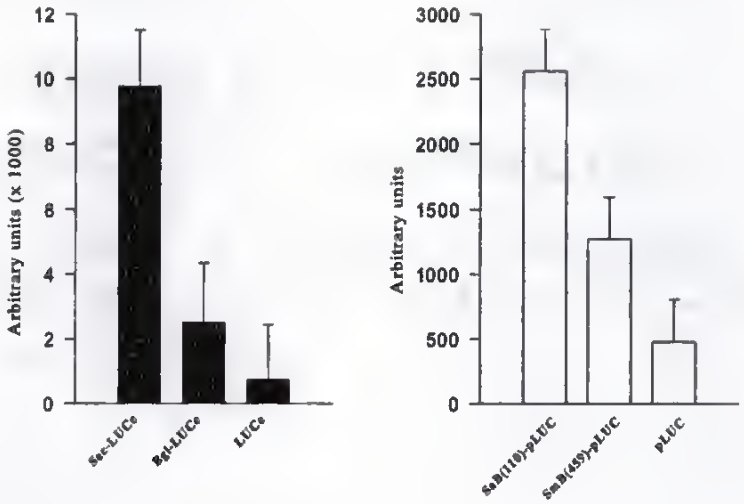


Figure 4-3. Transcriptional activation by the 110 bp upstream region. Bars represent the Least Squares Mean (n=9) (normalized by assay of cotransfected pCAT) of triplicate plates for each of three experiments (n=3 pigs). *Left* (black bars). Sac-LUCe had 4-fold higher activity than Bgl-LUCe ($p<0.05$), whereas there was no difference ($p=0.53$) between Bgl-LUCe and LUCe, in transfected endometrial glandular epithelial cells of Day 18 pregnant pigs. *Right* (open bars). SaB(110)-pLUC had 2 to 5-fold higher activity ($p<0.05$) than SmB(459)-pLUC and pLUC, respectively, whereas there was no difference ($p=0.16$) between SmB(459)-pLUC and pLUC, in transfected endometrial glandular epithelial cells from Day 18 pregnant pigs.

-874 to -765 into the pGL2-promoter vector in the sense orientation (Figure 4-1). This DNA was cotransfected with the pCAT plasmid (control for transfection efficiency) into primary cultures of GE cells from Day 18 pregnant pig endometrium. As controls, the SmB(459)-pLUC construct, which was generated by inserting the DNA fragment from -764 to -306 into the pGL2-promoter vector in the antisense orientation (Figure 4-1), and the pGL2-promoter plasmid (pLUC) were also transfected. These results (Figure 4-3) indicated that the 110 bp fragment increased SV40 promoter activity by approximately 5-fold, whereas the 459 bp fragment did not significantly increase the SV40 promoter's activity, in agreement with the previous results in Figure 4-2. These data supported the idea that positively-active cis-acting elements may exist within the 110 bp region and also showed that the activating function may not be promoter-specific.

Identification of Potential *cis*-Elements within the 110 bp Activating Region

As an initial step to examine for the binding of nuclear proteins to the 110 bp region, the DNA fragment generated by cleavage of Sac-LUCe with *Sac*I + *Bgl*II (Figure 4-4) was used in gel retardation assay of nuclear extract from endometrium of a Day 18 pregnant pig. This preliminary experiment confirmed that this fragment specifically bound nuclear proteins (Figure 4-5). Because of the relatively large size of this fragment, the binding complex theoretically could contain multiple proteins. Therefore to clarify the nature of this binding, the 110 bp region was subdivided into the A (40 bp), B (30 bp) and C (40 bp) subregions and used in GRA (Figure 4-4). Surprisingly, all three subregions bound to endometrial nuclear protein(s) with different patterns and multiple bands observed (Figure 4-5).

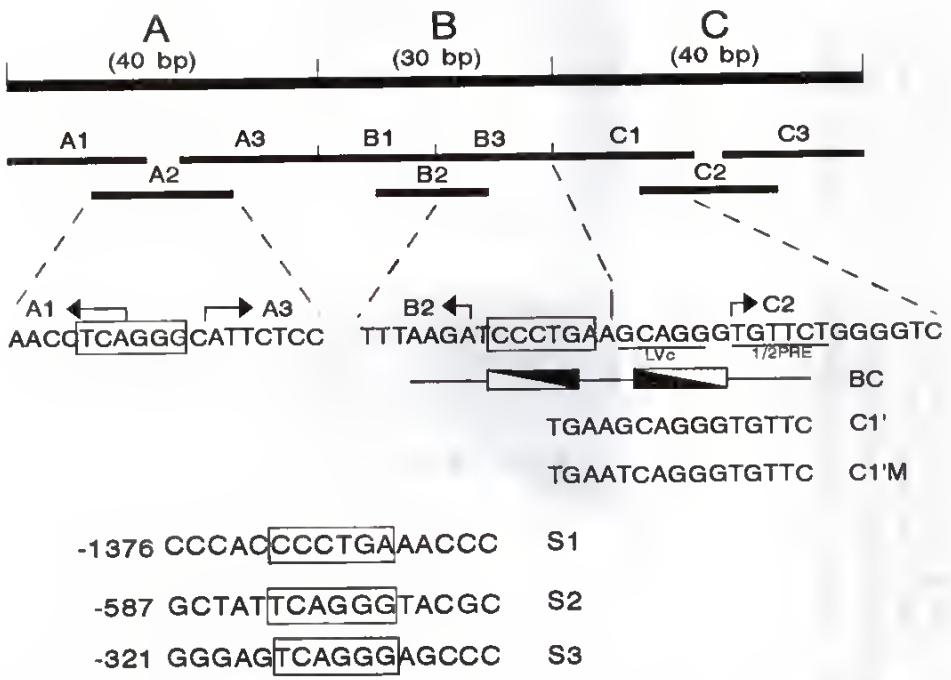


Figure 4-4. Map of the GRA probes derived from the 110 bp region. The top thick line represents the 110 bp fragment and the relative positions of the A, B and C probes. Probes A1, A2, A3, C1, C2, and C3 are 18 bp long. A2 has 7 bp on each end overlapping with A1 and A3. C2 has 7 bp on each end overlapping with C1 and C3. The probes B1, B2, and B3 are 15 bp long. B2 has 6 and 7 bp overlapping with B1 and B3, respectively. The A2 and B3 common sequence is boxed. The half progesterone receptor binding element (half site) is indicated as ½ PRE. The leukemia virus factor c (Lvc) binding element (Speck et al., 1987) is indicated. The probes BC, C1' and C1'M have overlapping sequences with B3 and C1. BC contains a palindromic sequence with a 3 bp spacer. C1' contain 4 bp of B3 and 11 bp of C1. C1'M has only one bp different from C1' at the 5th position from the 5' end. Three other oligonucleotide probes corresponding to TCAGGG-containing subregions (designated S1, S2 and S3) outside of the 110 bp region and flanking the IGFBP-2 promoter, are shown on the bottom. The location of each probe relative to the ATG translational initiation codon is indicated.

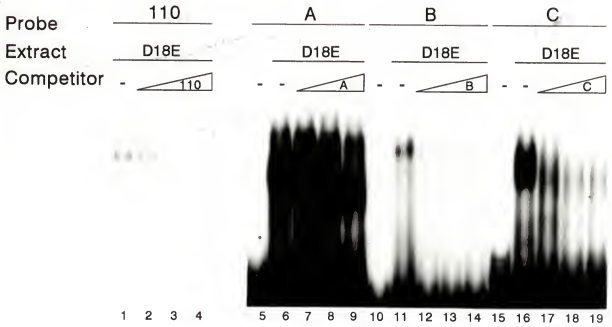


Figure 4-5. Uterine endometrial nuclear proteins bind the 110 bp fragment, and the individual A, B and C subregion probes. Using the gel retardation assay as described in Material and Methods, each probe was incubated with no nuclear extract (lanes 5, 10, and 15), with 10 μg of nuclear extract (lanes 1, 6, 11 and 16) or with 10 μg of nuclear extract preincubated with 50 X (lanes 2, 7, 12 and 17), 100 X (lanes 3, 8, 13 and 18) or 150 X (lanes 4, 9, 14 and 19) of a molar excess of unlabeled homologous competitor DNA, respectively.

In order to localize the individual protein-binding DNA elements, three overlapping double-stranded oligonucleotide probes within each of the A, B and C subregions were designed and synthesized (Figure 4-4). These probes were then used in GRA. Probes A2 and A3 competed with fragment A for binding to nuclear proteins; similarly probe B1 competed with fragment B; and probe C1 competed with fragment C (Figure 4-6). When all nine probes were individually labeled and used in GRA, the B2 and C3 probes were little or not shifted and therefore were eliminated from further study (Figure 4-7). Surprisingly, competition assays with the seven remaining probes showed that only the complex formed from the A2 probe and the largest complex formed from the B3 probe were completely inhibited by inclusion of excess unlabeled A2 and B3 oligonucleotides, respectively. All other complexes were not inhibited by excess unlabeled homologous competitor oligonucleotides and therefore were considered to represent nonspecific binding (Figure 4-8). The complexes identified with the A2 and B3 probes appear to have the same size as judged by co-migration in gels, suggesting that these two probes may bind the same protein. When the sequences of these two probes were closely examined, a common sequence 5'-TCAGGG-3' was found (reverse orientation in B3). To examine whether this common sequence in the A2 and B3 probes binds the same nuclear protein, ^{32}P-labeled A2 was used as probe and all nine unlabeled oligonucleotide probes were separately used as competitors in GRA. As predicted, only the A2 and B3 probes exhibited competition with labeled A2 (Figure 4-9), again suggesting that A2 and B3 bind the same nuclear protein herein designated as "A2 binding protein". Since A2 has 7 bp sequences overlapping with A1 and

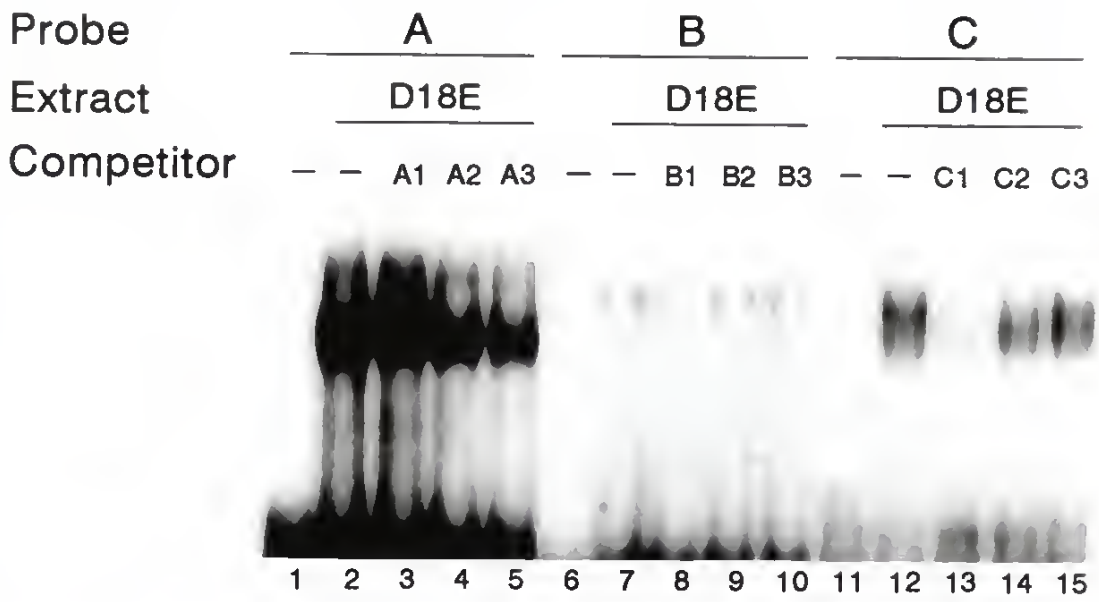


Figure 4-6. Localization of nuclear protein-binding sites within A, B and C subregions of the 110 bp fragment. Competitions of unlabeled A1, A2 and A3 oligonucleotides with A; Unlabeled B1, B2 and B3 oligonucleotides with B; and C1, C2, C3 oligonucleotides with C are shown. A 100 X molar excess of each competitor was preincubated with 10 µg of nuclear extract for 15 minutes prior to addition of the radiolabeled probe.

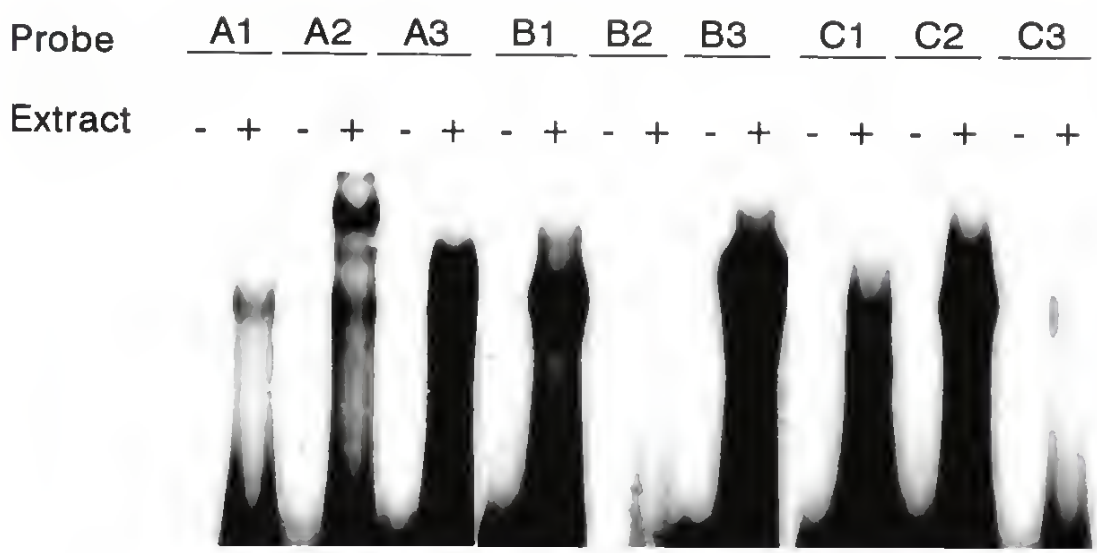


Figure 4-7. Gel retardation assay involving nine overlapping probes. Each probe was incubated without or with endometrial nuclear extract from a Day 18 pregnant pig. The gel for A1, A2 and A3 was exposed to X-ray film for 4 days, while the gel for B1, B2, B3, C1, C2 and C3 was exposed for 2 days.

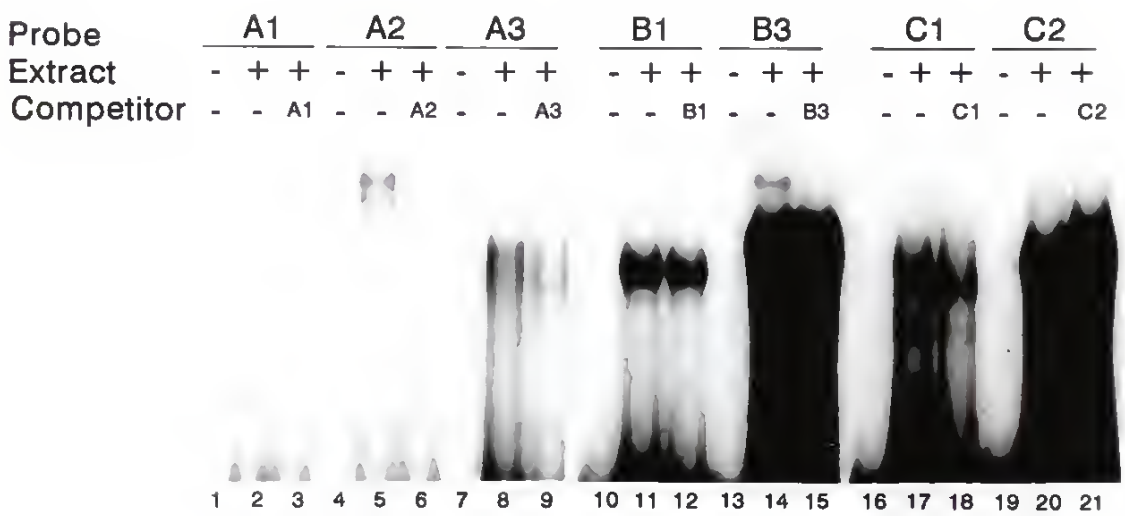


Figure 4-8. Localization of specific and non-specific protein-binding elements using GRA. Each probe was incubated without nuclear extract, with nuclear extract, or with nuclear extract which was preincubated with a 100 X molar excess of unlabed competitor oligonucleotide. The nuclear extract used in this experiment was from endometrium of a Day 18 pregnant pig.

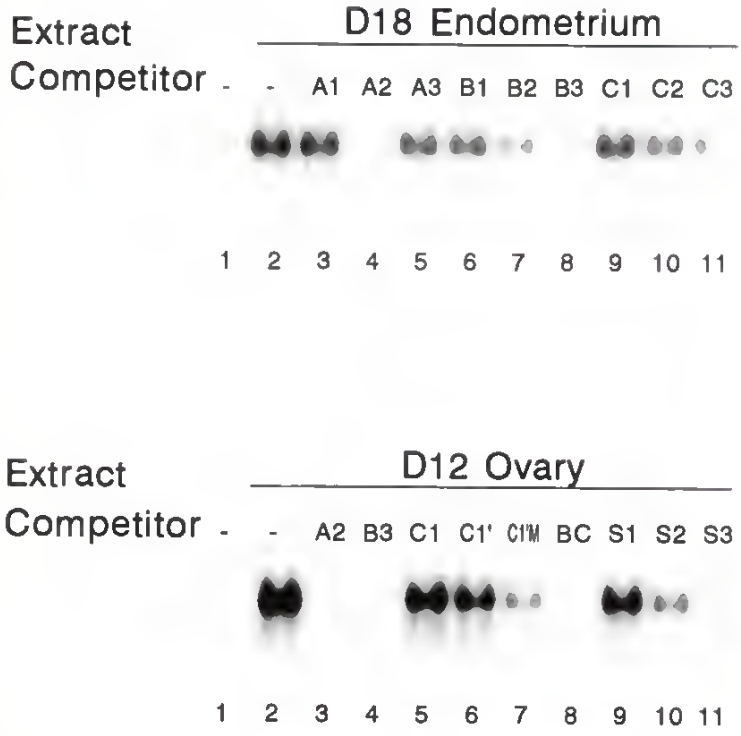


Figure 4-9. Proteins of the same molecular weight bind a common sequence within the A2 and B3 oligonucleotides. In both the top and bottom panels, a 50-fold molar excess of each unlabeled probe was used as competitor for binding of the A2 probe and nuclear extract prepared from the indicated tissue. Competitors used were defined in Figure 4-4.

A3 at both ends and B3 has 7 bp sequences overlapping with B2 at the 5'-end (Figure 4-4), the lack of competition by A1, A3 and B2 of A2-DNA binding suggests that the sequence 5'-TCAGGG-3, overlapping the middle of A2 and the 3'-end of B3 is, in all likelihood, the DNA binding element.

Using one base mismatch patterns at every position of the TCAGGG sequence to search the entire 110 bp region's sequence, two similar sequences were identified. One sequence, TCtGGG, is in the overlapping region of C1 and C2. The other sequence, gCAGGG, is at the 5'-end of C1. Since neither C1 nor C2 competed with A2 for binding, the sequence TCtGGG is judged to be non-binding, thereby suggesting that the A at the third position may be important for this interaction. However, the relative importance of the T at the first position of TCAGGG could not be inferred because this sequence is at the very end of probe C1. In this regard, it is possible that binding of proteins to this element requires flanking nucleotides. Moreover, a half PRE resides adjacent to this element (Figure 4-4). It is therefore possible that progesterone receptor interrupts the binding of this element to other nuclear proteins. The sequences CCCTGA in B3 and gCAGGG in C1 form a palindromic sequence 5'-CCCTGnnnCAGGG-3' with 3 bp spacer. To examine whether or not the gCAGGG element binds to the same protein as does A2, a new probe, C1' containing 4 bp at the 5' end of gCAGGG and 5 bp at the 3'-end but missing the half PRE (Figure 4-4), was designed and used as a potential competitor of A2 in GRA. C1' had little or no competition with A2 (Figure 4-9). In addition ^{32}P-labeled C1' was not shifted when incubated with nuclear extract (data not shown). These data therefore suggest that gCAGGG and its surrounding sequence has little or no interaction with A2 binding protein or other proteins,

such as Leukemia virus factor c (Lvc) (Figure 4-4) (Speck et al., 1987). The C1'M probe, which had the same sequence except for a 1 bp mutation from G to T (Figure 4-4) exhibited some competition with A2, but this was not as strong as for B3 and A2 (Figure 4-9). Thus the T at the first position of TCAGGG may be important for interaction with A2 binding protein. Moreover, the probe BC, which has both CCCTGA and gCAGGG competed for binding with A2 and exhibited the same size complex in GRA (Figure 4-10), again suggesting that the sequence CCCTGA (the reverse sequence of TCAGGG) is the only sequence interacting with the A2 binding protein.

Using computer-assisted sequence analysis, the sequence TCAGGG was found at 3 other positions in the sequenced 1.4 kb upstream region of the IGFBP-2 gene (Figure 4-4). Oligonucleotide probes corresponding to each of these regions were used as competitors in GRA. Only S2 and S3 competed with A2 but with differing affinities, whereas S1 exhibited little or no competition with A2 (Figure 4-9), suggesting that not only the TCAGGG but also the flanking nucleotides can affect interaction with the A2 binding protein.

Characterization of the A2 Binding Protein

In order to examine the tissue and possible stage of pregnancy-dependence of the A2 binding protein, nuclear extracts from endometria of Days 12, 18, 21, 60 and 90 pregnant pigs, placenta of a Day 90 pregnant pig and ovary of a Day 12 pregnant pig were used in GRA analysis. Data from this experiment showed that the A2 binding protein is present in all tissues examined (Figure 4-10). However, the relative binding in early pregnant pig endometrium was greater than in mid- or later-pregnant endometrium. The binding in the Day 12 ovary appeared to be the stronger. The ^{32}P-labeled A2 probe also was shifted by

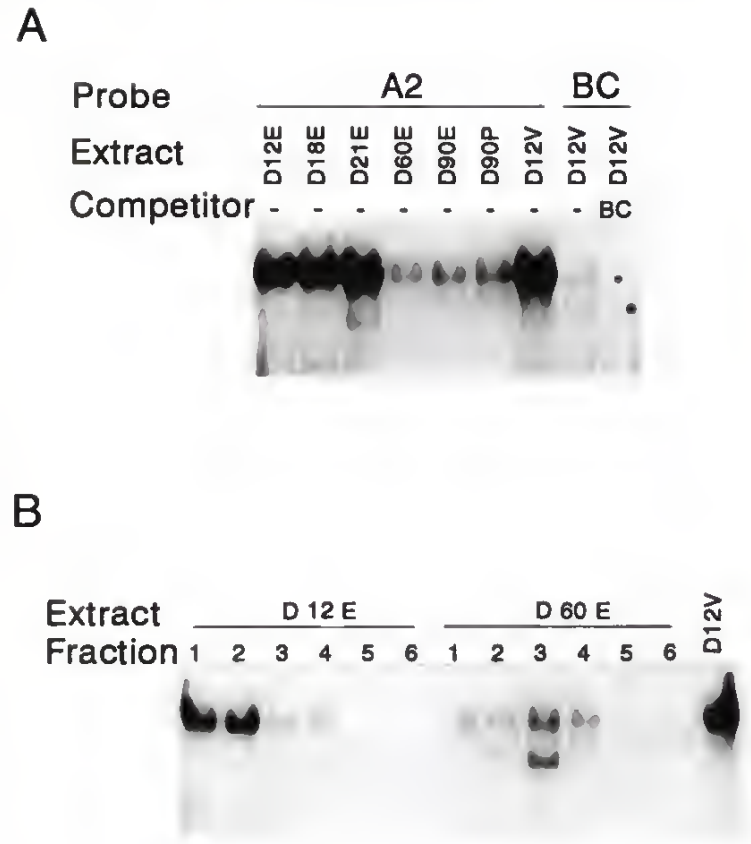


Figure 4-10. Characterization of the A2 element DNA binding protein. Panel A, nuclear extracts from Day 12 (D12E), Day 18 (D18E), Day 21 (D21E), Day 60 (D60E) and Day 90 (D90E) endometrium, Day 90 placenta (D90P), and Day 12 ovary (D12V) were used in GRA with radiolabeled A2 and BC probes. The right-most lane demonstrate the competition by a 100 X molar excess of unlabeled BC with the ^{32}P-labeled BC probe. Note that BC and A2 have the same size complex. Panel B, nuclear extracts from Day 12 and Day 60 endometria were fractionated on DEAE Bio-Gel A columns (see Materials and Methods). Fraction 1 is the flow-through prior to the application of the salt gradient. Fractions 2 to 6 were eluted in a salt gradient (0.1 M to 1.0 M). The nuclear extract from ovary of a Day 12 pregnant pig (D12V) was used as positive control. Probe A2 was used in all lanes of this GRA.

nuclear proteins from the (human hepatocarcinoma) HepG2 cell line with two smaller complexes apparent (data not shown). These same complexes were also observed in GRA analysis of some endometrial nuclear extract preparations.

When nuclear extracts from Day 12 and Day 60 endometria were fractionated on DEAE BioGel A columns and analyzed by GRA, two observations were made. First, A2 binding protein was present in Fraction 2 of Day 12 endometrial nuclear extract, whereas A2 binding protein was in Fraction 3 of the Day 60 endometrial nuclear extract (Figure 4-10). This delay in elution may indicate that the A2 binding protein in mid-pregnancy endometrium has more net negative charge than the corresponding protein in Day 12 endometrium. This increase in negative charge is possibly due to protein phosphorylation. Second, smaller A2 binding complexes were observed in some fractions of both the D12 and Day 60 endometrial nuclear extracts. However, these smaller complexes were most abundant in Fraction 3 of the Day 60 endometrial nuclear extract.

In order to characterize the A2 binding protein as to its molecular weight, UV cross-linking was employed. ^{32}P-labeled A2 oligonucleotide and nuclear proteins from ovary of Day 12 and endometrium of Day 18 of pregnancy were utilized in this experiment. Three cross linked products with sizes of 35 to 40 kDa were observed (Figure 4-11A). Similarly, Southwestern blotting (Figure 4-11B) showed that the A2 probe bound to a 33 kDa protein in nuclear extract from Day 12 ovary, Day 90 placenta, and in Fraction 2 of Day 12 endometrial extract. The specificity of this binding was also confirmed using competitive Southwestern blot with Fraction 2 of Day 12 endometrial nuclear extract (Figure 4-11C).

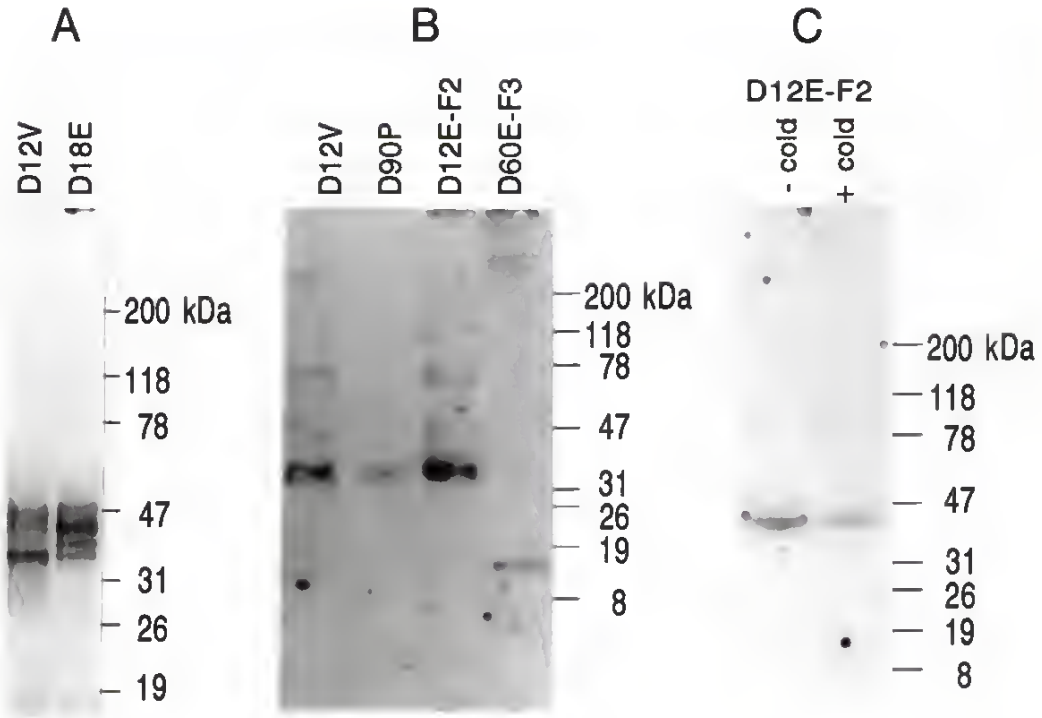


Figure 4-11. Size determination of the A2 DNA binding protein. A, UV cross-linking. Nuclear proteins from D12 ovary (D12V, 20 μg) and from Day 18 endometrium (D18E, 10 μg) of pregnant pig, were used in the assay as described in Materials and Methods. B. Southwestern blot. Nuclear proteins from Day 12 ovary (D12V, 100 μg), Day 90 placenta (D90P, 50 μg), Fraction 2 from Day 12 endometrium (D12E-F2, 200 μg) and Fraction 3 from Day 60 endometrium (D60E, 100 μg) of pregnant pigs were used in the assay as described in Materials and Methods. C. Competitive Southwestern blot. Nuclear proteins in Fraction 2 from Day 12 endometrium (D12E-F2, 175 μg) was used for each lane. The left lane was preincubated with a 50 x molar excess of cold A2 probe. Protein size markers are indicated on the right side of each gel.

However, in Fraction 3 of the Day 60 nuclear extract, the A2 probe bound to a 13 kDa protein (Figure 4-11B).

Discussion

Although the 5' flanking sequence of the IGFBP-2 gene has been isolated for the human (635 bp, Binkert et al., 1992), rat (1290 bp, Kutoh et al., 1993), murine (1980 bp, Landwehr et al., 1993), chicken (661 bp, Schoen et al., 1995) and porcine (1397 bp, Chapter 2), few studies on the functions of this region have been conducted. Schwander and colleagues, took the -579 to +1 (ATG, +1) fragment of the rat IGFBP-2 gene, and using deletion and transfection analysis followed by gel retardation assay demonstrated the presence of three GC boxes that bind transcription factor SP1 and display cooperativity in transcriptional assays (Kutoh et al., 1993). Similarly, Rechler and colleagues also demonstrated that these three SP1 sites are required for efficient transcriptional initiation of the rat IGFBP-2 gene (Boisclair et al., 1993). Recently, Schwander and colleagues showed that cell density can affect IGFBP-2 gene expression and similarly affects the promoter activity of a 1.5 kb flanking fragment of the rat IGFBP-2 gene (Kutoh et al., 1995). All of these studies mainly used rat BRL-3A liver cells. To date no comparable studies have been conducted on other species or tissues. In this regard, SP1 gene and protein expression in pregnant pig endometrium is constitutively low and is not correlated with the temporal pattern of endometrial expression of IGFBP-2 gene (Wang, Y. and Simmen, R.C.M., unpublished observations). Moreover, potential distal sequence contributions to the transcriptional regulation of this gene, in concert with or independent of SP1, were not previously studied.

As an extension of previous work on the tissue and developmental expression and characterization of IGFBP-2 gene, the present study examined the distal region flanking the IGFBP-2 promoter for transcription regulation. Transient transfection assays using two cancer cell lines (ECC-1 and JEG-3) and primary cultures of pig endometrial cells (GE and ST) at two different pregnancy stages, indicated that deletion from -874 to -765 (Sac-LUCe vs Bgl-LUCe) decreased the reporter gene activity. Fusion of this region to the SV40 promoter, increased the promoter activity by 5-fold, clearly demonstrating that the 110 bp region from -874 to -765 has transcriptional stimulatory activity. Moreover, this stimulatory activity has also been observed in HepG2 cells (Badinga, L., Song, S., Simmen, F.A., and Simmen, R.C.M., unpublished data). Thus, this stimulatory activity appears not to be cell type- or developmental stage-specific. The position- and orientation- dependence of this stimulatory activity has yet to be examined. However, results demonstrate that this stimulatory activity is functional for two different promoters.

Transfection experiments also suggested that regions from -1397 to -875 and -764 to -306 have transcriptional repressing activities, which appear to be cell-type and/or developmental stage-specific. In Ecc-1, JEG-3 and D18 ST cells, the region from -764 to -306 (459 bp) exhibited a strong repressing activity (B vs Sm). This repressing activity was also noted in D30GE cells, although this inhibitory activity was not statistically significant. However, transfected D18GE and D30ST cells exhibited no repressive effect with the -764 to -306 construct. In D18GE cell, this region did not decrease SV40 promoter activity again suggesting no repressing affect in this cell type.The inhibitory activity of the region from -1397 to -875 (523 bp) was manifested in ECC-1 cells and in all pig endometrial cell cultures,

but was not observed in JEG-3 cells. Moreover, in HepG2 cells, this region has transcriptional stimulatory activity (Badinga, L., Song, S., Simmen, F.A., and Simmen, R.C.M., unpublished data). These observations taken together suggest that this 523 bp region exhibits cell type-dependent regulatory activity. However, these two regions remain to be further investigated and may prove useful for the eventual identification of tissue and developmental regulatory *cis*- and *trans*-elements required for IGFBP-2 gene expression.

Transfection data from all cell types showed that the Sma-LUCe construct has relatively high and constant reporter gene activity (about 5000 arbitrary luciferase light units). This observation may indicate that the region from -305 to +73 covers the basal promoter region, which is controlled by ubiquitous transcription factors, such as SP1 (Faisst and Meyer, 1992; Kutoh et al., 1993; Boisclair et al., 1993; Y . Wang and R.C.M. Simmer, unpublished observations).

There were two reasons why primary cultures of endometrial cells were used for the present study. First, there are no continuous pig endometrial cell lines available. Second, primary cell cultures are perhaps closer to the normal physiological condition, which may be important for transcriptional activity and regulation of the IGFBP-2 gene. The IGFBP-2 gene is highly expressed in Days 30 and 60 pregnant pig endometrium, and is expressed at much lower levels in Day 12 pig endometrium (Simmen et al., 1992; Chapter 3). In this regard, Day 60 GE cells may be the best cell type for identification of IGFBP-2 gene regulatory region(s). However, Day 60 GE cells did not survive the electroporation process, and consequently reporter gene activity was very low (data not shown). In the pig, placentation initiates around Days 14-16 of pregnancy (Dantzer et al., 1985; Keys and King,

1990). At this time, signals from the embryo are thought to trigger the temporal expression of a battery of uterine genes (Roberts et al., 1993; Simmen et al., 1995). Since IGFBP-2 gene expression is induced by Day 18 of pregnancy, a similar embryo-maternal signaling mechanism may be involved. In the present study, glandular epithelial and stromal cells from both Day 18 and Day 30 of pregnancy were successfully used for transfections. However, GE cells from Day 18 pregnant pig endometrium exhibited the highest reporter gene activity after DNA transfection.

Using nine overlapping oligonucleotide probes, two potential *cis*-elements TCAGGG and their common binding protein were identified within the 110 bp transcriptional inducing region. Although mutation on each position for the consensus element has not been done, two native mutants TcTGGG and gCAGGG in the 110 bp region did not show competition with A2, suggesting that "G" at the first and "T" at the third position from the 5'-end are important for binding. This TCAGGG sequence was also found at other positions (S1, S2 and S3). However, different degree of competition with A2 were observed, suggesting that flanking sequences at each end of this element are also important for protein binding.

It remains possible that there are other important *cis*-elements within this 110 bp region. Although the competition study with nine oligonucleotide probes showed incomplete self competition by all probes except for A2 and B3, partial self competitions by A3, B1 and C1 oligonucleotides were observed. A3, B1 and C1 also exhibited some competition with A, B and C, respectively. These data may indicate that the binding affinities are low under conditions presently used. Collaborative studies (Badinga, L., Song, S., Simmen, F.A., and Simmen, R.C.M., unpublished data) have shown that in HepG2 human liver cells, A3 and

C3 bind to a common protein via a consensus sequence CANNTG. Moreover, the break points for each of these probes may interrupt a protein binding. Therefore, other *cis*-elements remain to be identified and which possibly function in other cell types or under other physiological conditions.

The sequence TCAGGG identified here was first recognized as TCAGGN in the conserved promoter region of histone H4 genes (Clerc et al., 1983). Recently, it has been reported that TCAGGG and TGAGGG, as variant telomere TTAGGG repeats, are distributed at the proximal end of the PAR1 telomere, which is in the human Xp/Yp pseudoautosomal region (Baird et al., 1995). However, no data are available for the corresponding trans-acting factors that bind to this sequence. Data from this study are the first to identify a nuclear protein with molecular weights of 33 and 13 kDa that specifically interacts with this sequence. Gel retardation assays using nuclear extracts from different tissues showed that the A2 DNA binding protein is present in all tissues examined, including endometrium, placenta, ovary and HepG2 cells (data not shown), suggesting that this nuclear protein is ubiquitously expressed. Although gel retardation assays may not be sensitive enough for quantification of this protein, the binding interaction was lower in D60E (high IGFBP-2 expressing tissue) than in D12E and D90P (low or no IGFBP-2 expressing tissues).

By UV cross-linking, the cross linked product was estimated to be 35 kDa in size. This closely agreed with 33 kDa size estimated by Southwestern blot, if the size contribution due to the oligonucleotide probe (A2) in the cross linked product is considered. The larger cross linked products may indicate interactions between the 33 kDa A2 element-binding protein and other nuclear proteins before UV-cross linking. Similarly, the larger bands in

Southwestern blot (Figure 4-11B) may represent the incompletely denatured A2 DNA binding proteins such as a dimer, since this band was not observed in competitive Southwestern blots. However, the nature and identity of this 33 kDa nuclear protein remain to be further investigated.

The observation of a 13 kDa A2 element-binding protein in Fraction 3 of nuclear extracts from Day 60 pregnant pig endometrium indicated two possibilities. First, the 13 kd A2 binding protein may be a truncated form of the 33 kDa protein. Second, the 13 and 33 kDa proteins may be different proteins. Nevertheless, the 13 kDa nuclear protein may be important for the transcriptional activity or regulation of the IGFBP-2 gene, since this gene is highly expressed at mid-pregnancy. Moreover, since the 13 kDa protein appeared in Fractions 3 or 4 from nuclear extracts of both Day 12 and Day 60 pregnant pig endometria, phosphorylation of this protein may occur, perhaps in parallel with the 33 kDa protein, which may be important for its regulatory activity.

Summary

IGF-I and -II play important roles in fetal and maternal interactions. IGF binding proteins (IGFBPs), a family of at least six members, are important modulators of IGF actions. In pig uterus, IGFBP-2 has a relatively unique pattern of expression. However, the molecular mechanisms of transcriptional regulation of this gene in uterine tissues are unknown. The present study was undertaken to identify potential functional upstream regulatory regions and corresponding *cis*-elements as well as *trans*-acting factors that interact with these *cis*-elements. Transient transfection in different cell lines (ECC-1 and JEG-3) and uterine endometrial cell types (glandular epithelial and stromal cells) identified an upstream

110 bp region from -874 to -765 with transcriptional enhancing activity. This activity was found to be neither cell type-specific nor promoter-specific. A series of gel retardation assays identified two consensus sequences TCAGGG and CCCTGA in this 110 bp region that bound the same endometrial nuclear protein herein named the A2 DNA binding protein, which has a molecular weight of 33 kDa. This protein is present in endometria of pigs at all pregnancy stages as well as in placenta, ovary and human HepG2 liver cells. In the endometrium of Day 60 pregnant pigs, this protein has greater net negative charge, suggesting differential phosphorylation of this protein during pregnancy. The widespread distribution of the A2 element DNA binding protein was in agreement with the apparent stimulatory activity of the 110 bp region in different cell types.

CHAPTER 5
STEROID AND PEPTIDE HORMONAL REGULATION OF UTERINE IGFBP-2 GENE TRANSCRIPTION

Introduction

Expression of the IGFBP-2 gene exhibits tissue-, temporal- and developmental-specific changes during pregnancy (Simmen et al., 1992; Song et al., 1996). A number of studies have demonstrated expression of this gene in a cell-type specific manner (for review see Chapter 2). Moreover IGFBP-2 functions in an endocrine as well as paracrine and autocine manner to affect cell proliferation, apoptosis and perhaps differentiation. As a member of the IGF system, IGFBP-2 may be directly or indirectly controlled by other hormonal and trophic factors.

Estrogen and progesterone have potential regulatory roles in uterine endometrial expression of the IGFBP-2 gene. As major regulatory factors for uterine secretory function, these two steroid hormones may directly or indirectly regulate gene transcription through their nuclear receptors, which require the presence of defined *cis*-elements (ERE and PRE) for their actions. It was previously reported that estrogen and progesterone or EGF and progesterone stimulates uterine IGFBP-2 mRNA production and that IGFBP-2 mRNA in secretory phase endometrium (under the influence of progesterone) was 2.1-fold higher than in proliferating endometrium which is under the influence of estrogen (Giudice et al., 1991,

1992). In cyclic bovine endometrium, maximal IGFBP-2 mRNA abundance was observed at the mid-luteal phase (low estrogen and high progesterone) (Geisert et al., 1991). In cyclic pig endometrium, however, the abundance of IGFBP-2 mRNA was temporally correlated with maximal concentrations of estrogen and negatively correlated with progesterone concentration in the circulation (Simmen at al., 1992). In pregnant pig endometrium, maximal IGFBP-2 mRNA was observed at mid-pregnancy (rising estrogen, declining progesterone) (Chapter 3 and Simmen et al., 1992). During pregnancy in the equine, IGFBP-2 mRNA abundance was temporally correlated with maximal serum estrogen and declining serum progesterone (Simmen, F. A., unpublished observations). The collective results from these studies suggest but do not prove that estrogen and progesterone regulate uterine IGFBP-2 gene activity. However, in different species, this presumed regulation appears to be different. A previous study (Chapter 3) identified several candidate EREs and PREs in the 1.4 kb flanking region of the IGFBP-2 gene. In the present study, estrogen and progesterone were directly examined as candidate regulatory hormones for IGFBP-2 gene expression in uterine cells.

Among the IGFBPs, IGFBP-2 is perhaps the least intensively studied with regard to transcriptional regulation. Cazals et al. (1994) showed that in rat lung alveolar epithelial cells, TGF-β1 induced by oxygen exposure was a potential stimulator of IGFBP-2. It was also reported that, IGFBP-2 expression in rat embryos was highly correlated with IGF-II expression at all ages from early post-implantation through to mid-gestation (Wood et al., 1992). IGF-I and IGFBP-2 mRNA abundance were inversely correlated in pig endometrium and myometrium during pregnancy (Song et al., 1996). Moreover maximal IGFBP-2 mRNA

abundance corresponded to high IGF-II mRNA abundance in endometrium, placenta and fetal tissues of the pregnant pig. However, there is as yet no direct evidence to demonstrate an effect of any peptide or steroid hormone on IGFBP-2 gene expression in endometrial cells. In this chapter, TGF-β1, IGF-I, IGF-II, Phorbol 12-Myristate 13-Acetate (PMA) and cAMP were examined for their possible effects on IGFBP-2 gene expression using cultured endometrial glandular epithelial cells from Day 18 pregnant pigs, a model system established in the previous Chapter for studying IGFBP-2 gene regulatory mechanisms.

Materials and Methods

Animals

Gilts were monitored twice daily for onset of estrous activity. Gilts exhibiting two consecutive estrous cycles of normal duration (18-22 days) were mated at estrus with boars and again 12 and 24 h later. The day of onset of estrus was defined as Day 0 of pregnancy. Animals were sacrificed at the University abattoir on the indicated days of pregnancy. Reproductive tracts were removed, immersed in ice and trimmed from the mesometrium. Endometria, myometria and placentae were obtained by dissection as previously described (Simmen et al., 1990; Ko et al., 1994a). Animal use protocols were approved by the University of Florida Institutional Animal Care and Use Committee.

Ovariectomies

On Day 4 of the estrous cycle, twelve sexually mature, crossbred gilts were subjected to bilateral ovariectomy as described previously (Buhi et al., 1992). Gilts were randomly divided into four treatment groups (three for each): 1) control (C) group treated with corn oil/ethanol as vehicle; 2) Estrogen (+E2) group treated with 100 μg estradiol valerate; 3)

progesterone (+P4) group treated with 200 mg progesterone; 4) Estrogen and progesterone group (+EP) treated with 100 μg estrogen and 200 mg progesterone. Treatments were administered intramuscularly for 11 consecutive days and hysterectomies were performed on day 15. Endometrial tissue was collected and snap frozen in liquid nitrogen.

Cell Cultures

Primary cultures of uterine cells were obtained as described previously (Zhang et al. 1994; Reed et al., 1996). In brief, the uterus was removed from a pregnant gilt and flushed with Phosphate-Buffered Saline (PBS) containing 1% ABAM (Antibiotic Antimycotic: 500U/ml penicillin, 500 μg/ml streptomycin, 25 μg/ml amphotericin B). Endometrium was obtained by dissection and washed 3 times with Hank's Balanced Salt Solution (HBSS) (Gibco BRL, Life Technology, NY) also containing 1% ABAM and 50 ug/ml gentamicin. The tissue was incubated in digestion solution I (4.8 mg/ml dispase and 12.5 mg/ml pancreatin in HBSS) for 2.5 hours at room temperature . The tissue was then washed a total of 6 times in HBSS containing 1% ABAM to obtain luminal epithelial cells. The remaining tissue remnants were incubated in digestion solution II (0.04% trypsin, 0.06% collagenase, 0.01% DNase I in HBSS) for 50 minutes at 37 °C to dissociate into a single cell suspension. Glandular epithelial cells and stromal cells were separated by passage of the resultant cell suspension through a 38 um stainless steel sieve. Glandular epithelial cells were seeded in 35 mm well, 6-well plates in RPMI 1640 medium also containing 10% fetal bovine serum (FBS) and 1 % ABAM. Medium was changed every two to three days until the cells were ready for DNA transfection or hormonal treatments.

Charcoal stripped serum was prepared as follows: 0.25 % Norit A charcoal (Sigma Chemical Co.) and 0.0025 % dextran T-70 (Pharmacia) were incubated overnight at 4 °C in 0.25 M Sucrose, 1.5 mM $MgCl_2$, 10 mM Hepes, pH 7.4. The dextran-coated charcoal was recovered by centrifigation (500 xg for 10 min) and resuspended in an equal volume of fetal bovine serum (FBS). The mixture was incubated overnight at 4 °C and then centrifuged to remove the charcoal. Serum was stored at -20 °C.

RNA Isolation

Total cellular RNA was isolated from endometrial tissues by acid guanidinium thiocyanate-phenol-chloroform extraction (Puissant and Houdebine, 1990). Polyadenylated RNA was enriched from total cellular RNA by batch affinity chromatography on oligo(dT)-cellulose (Celano et al., 1993). Total RNA was extracted from cultured cells using TRIzol™ reagent (Gibco BRL, Life Technologies Inc.)

RNase Protection Assay

Plasmid BP2-18 (see Chapter 3) was cleaved with *Sma*I. The fragment containing the SP6 promoter and the DNA insert, spanning from +73 to -305, was purified by agarose gel electrophoresis and DEAE paper, and used as template for synthesis of probe for RNase protection. The ^{32}P-labelled antisense probe was synthesized using the MAXIscript™ SP6/T7 *in vitro* transcription Kit (Ambion Inc., Austin, Texas). RNase protection assays were performed using the HybSpeed™ RPA Kit (Ambion Inc.). Products of the RNase protection assay were resolved in 6% denaturing, polyacrylamide gels.

Transfection of Primary Cultures

Cells were detached from plates by treatment with trypsin and collected by centrifugation at 2000 rpm for 3 min. The cell pellet was resuspended in transfection buffer [Hepes Buffered Solution (HBS): 21mM Hepes, pH, 7.05, 137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, 6 mM Glucose]. Luciferase reporter plasmids (20 μg) were transfected by electroporation (Cell-Porator, GIBCO BRL, Life Technologies, Inc., setting at 200-250 voltage and 330 μF) at room temperature. The transfection efficiency was normalized for protein content of cell extracts or for pCAT activity determined after co-transfection of pCAT plasmid (10 g) (Promega, Corp., Madison, WI). Forty eight hours post-transfection or addition of treatments, cells were washed 2 times with PBS, and lysed in 200 μl of cell lysis buffer (Promega, Corp., Madison, WI). One hundred μl of cell extract was saved for protein or CAT assay. Another hundred μl was centrifuged at 12000 rpm for 5 min. Thirty μl of supernatant was used to determine Luciferase activity(AutoLumat LB953 luminometer, EG&G Berthold) for 3 min using luciferase assay substrate (Promega Corp., Madison, WI). Protein concentration was determined using the Bradford method with bovine serum albumin as the standard.

For CAT assays, 100 μl of cell extract was incubated at 65 °C for 10 min and centrifuged at 12000 rpm for 10 min. Eighty μl of supernatant was adjusted to a volume of 125 μl buffer in 0.25 mM Tris-HCl, pH 8.0 containing 0.08 μCi ^{14}C chloramphenicol and 25 μg n-butyryl CoA, and incubated at 37 °C for overnight. After extractions with mixed xylenes and a back extraction with 0.25 mM Tris-HCl (pH 8.0), 200 μl of xylenes containing

the radiolabeled CoA was placed in a liquid scintillation counter for quantitation of radioactivity.

All transfection data were analyzed by least squares analysis of variance using the General Linear Models-Procedures of the Statistical Analysis System (SAS; Barr et al. 1979). The effects of experiment, pig (as random effect), DNA constructs and their interactions were considered in the model. The protein content or pCAT activity was used as a covariate to normalize the luciferase activity. Differences between constructs were examined using preplanned orthogonal contrasts.

Ligand Blot Analysis

The procedure used was described by Mathieu et al. (1990). Briefly, cell membranes were solubilized in 0.33 x RIPA lysis buffer (Burr et al., 1980) (1x=0.1 M sodium phosphate, pH 7.2, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 mM EDTA, 10 mM EGTA, 10 mM NaF, 1% (w/v) SDS and 200 Kallikrein inhibitor units of aprotinin/ml) for 1 h at 4 °C with shaking. The insoluble material was removed by brief centrifugation. Samples were boiled for 3 min and subjected to 7.5% SDS-PAGE under nonreducing conditions and transferred to a nitrocellulose membranes. The membrane were incubated in a buffer containing 10 mM Tris, 0.9% NaCl, 0.1% Tween 20, 0.3% Triton X-100, 0.2% BSA, 1% nonfat dry milk, and 0.1% NaN_3 for 24 h at 4 °C and then incubated with [^{125}I]IGF-II (80,000 cpm/ml) in binding buffer (25 mM Tris, pH 7.4, and 10 mM $MgCl_2$) for 3 h at room temperature. The membrane was washed with 20 mM Tris, pH 7.4, containing 10 mM $MgCl_2$, and 0.1% Tween 20 (2x15 min) and then with 20 mM Tris, pH 7.4, containing 10 mM $MgCl_2$, for 20 min.

Results

Effects of Estrogen and Progesterone on Uterine Endometrial IGFBP-2 Gene Expression

In vivo - ovariectomized pig model. Ovariectomized pigs were treated with vehicle, estrogen, progesterone or the combination of estrogen and progesterone. Endometrial poly $(A)^+$RNAs were extracted from these tissues and subjected to RNase Protection Assay (RPA) for lGFBP-2 exon 1 containing transcripts. Data obtained from three pigs for each group indicated (Figure 5-1) that estrogen or estrogen plus progesterone increased lGFBP-2 mRNA abundance by ~5-fold ($p<0.02$) or 6-fold ($p<0.01$) in ovariectomized pigs. Effects of progesterone were also statistically significant ($p<0.07$).

In vitro - primary cultures of endometrial glandular epithelial cells. Glandular epithelial cells were isolated from endometrium of Day 18 pregnant pigs (D18GE) and cultured in RPMI 1640 medium supplemented with 10 % FBS for 3 to 5 day until the cells reached ~80% confluence. These cells were then placed in medium containing 10 % charcoal stripped serum for 24 hours. At this point the medium was supplemented with vehicle (PBS and ethanol), 1 nM estrogen (+E2), 25 nM progesterone (+P4), or 1 nM estrogen plus 25 nM progesterone (+EP). All cells were then incubated for an additional 24 hours. RNA was extracted from these cells and subjected to RPA for lGFBP-2 mRNA. Results from this experiment showed that estrogen stimulated lGFBP-2 mRNA abundance ($p<0.01$). However, no effect of progesterone was observed ($p=0.38$). The effects of estrogen vs. estrogen plus progesterone were not statistically different again suggesting no effect of progesterone in this primary cell culture system (Figure 5-1).

Effects on IGFBP-2 Promoter Activity. The Sac-LUCe and Bgl-LUCe constructs were transfected into D18GE cells which were then incubated in RPMI 1460 medium containing charcoal-stripped serum for 24 hours. At this point, the medium was supplemented with 1 nM estrogen, 25 nM progesterone, or 1 nM estrogen plus 25 nM progesterone and the cells incubated for an additional 24 hours. As evident in Figure 5-2, progesterone with or without estrogen decreased the reporter gene activity of the Sac-LUCe plasmid ($p<0.02$), while estrogen effect was not significant ($p=0.18$). In Bgl-LUCe transfected GE cells, treatment with estrogen ($p=0.21$), progesterone ($p=0.22$) did not significantly alter the reporter gene activity. Results from these experiments therefore suggest that progesterone may inhibit the transcription of the IGFBP-2 gene in this system via the upstream activating 110 bp region.

In order to confirm this negative effect, the construct Sac-LUCe was transfected into D18GE cells. Transfected cells were incubated in RPMI 1640 medium containing 10 % charcoal stripped serum for 48 hours. Within this time period, 1 nM estrogen or 25 nM progesterone was added at 1, 6, 12, or 24 hours prior to the end of the 48 hour period. Results from this experiment showed that both estrogen and progesterone decreased promoter activity in a time-dependent manner (Figure 5-3).

When the construct SaB(110)-pLUC in which the 110 bp fragment from -874 to -764 is fused to pGL2-promoter vector (see Chapter 4), was transfected into D18GE cells, treatment with estrogen or progesterone did not significantly affect the promoter activity (Figure 5-4) suggesting that the above described estrogen and progesterone inhibitory effects require the presence of the downstream region (from -764 to +73).

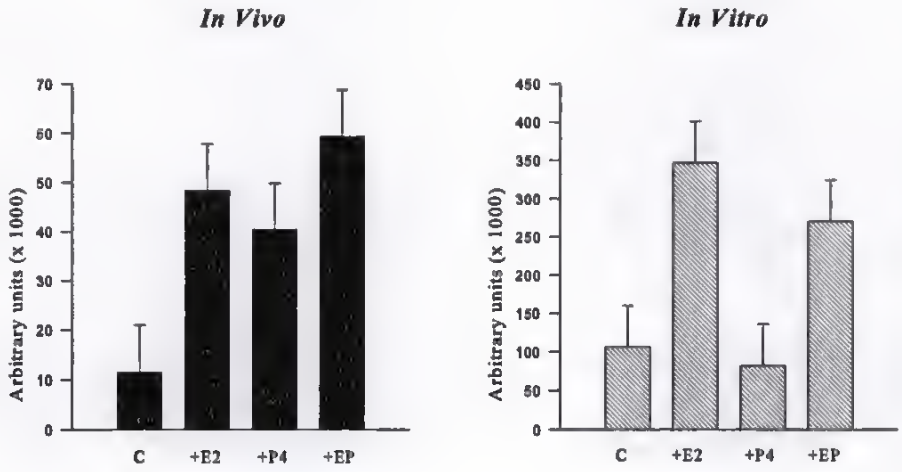


Figure 5-1. Effects of estrogen and progesterone on IGFBP-2 steady-state mRNA abundance *in vitro* and *in vivo*. Each bar represents the least squares mean ± SEM of phosphor imaging of data from RPA. The Y-axes are arbitrary units. Left panel, RNA was extracted from endometria of ovariectomized pigs (n=3 pigs for each treatment group), which were untreated or received estrogen (+E2), progesterone (+P4) or estrogen plus progesterone (+EP). Four μg of poly $(A)^+$ RNA from each individual pig was subjected to RPA. Right panel, RNAs were extracted from primary cultures of porcine endometrial glandular epithelial cells (n=3 plates for each treatment group), which were incubated in RPMI 1640 medium containing charcoal-stripped fetal bovine serum (CS, control group) and supplemented with 1 nM estrogen (+E2), 25 nM progesterone (+P4) or estrogen plus progesterone (+EP) for 24 hours. Fifteen μg of total RNA was subjected to RPA.

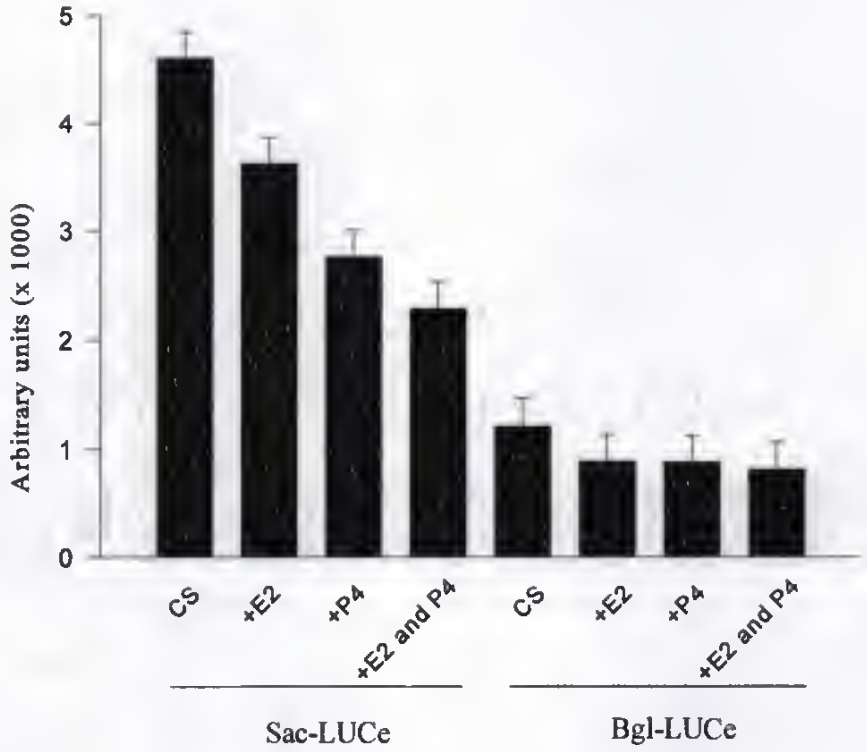


Figure 5-2. Effects of estrogen and progesterone on the promoter activities of Sac-LUCe (-874/+73) and Bgl-LUCe (-764/+73) DNA constructs in primary cultures of transfected endometrial glandular epithelial cells from Day 18 pregnant pigs. Each bar represents the Least Squares Mean ± SEM (n=9) in arbitrary luciferase light units (normalized for cotransfected pCAT activity) of triplicate plates for three independent experiments (n=3 pigs). Transfected cells were incubated in RPMI 1640 medium containing charcoal-stripped fetal bovine serum and supplemented with vehicle (CS), 1 nM estrogen (+E2), 25 nM progesterone (+P4) or the combination of estrogen progesterone (+EP) for 24 hours.

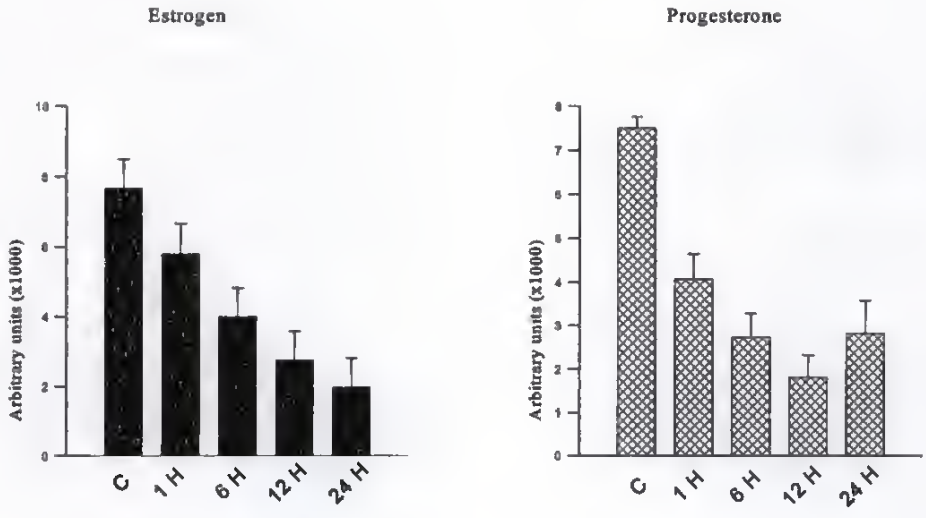


Figure 5-3. Effects of estrogen and progesterone on reporter gene activity of the Sac-LUCe (-874/+73) DNA construct in a time-course experiment. Each bar represents the Least Squares Mean ± SEM (n=9) in arbitrary luciferase light units (normalized for cotransfected pCAT activity) of triplicate plates from three independent experiments (n=3 pigs). After transfection, cells were incubated in RPMI 1640 medium containing charcoal-stripped fetal bovine serum and supplemented with vehicle, 1 nM estrogen (black bars), or 25 nM progesterone (stippled bars) for 1, 6, 12 and 24 hours. For all cell cultures, the total time of incubation post-transfection was 48 hours.

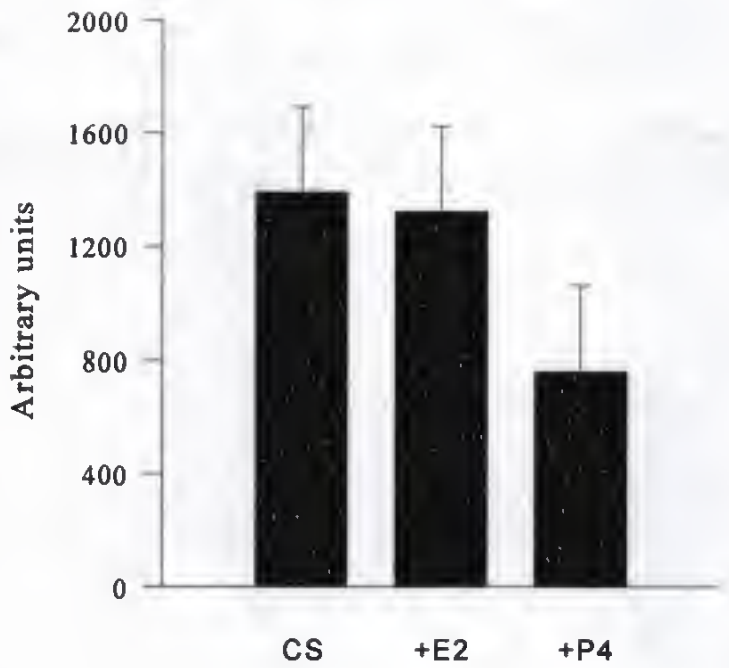


Figure 5-4. Effects of estrogen and progesterone on promoter activity of SaB(110)-pLUC (-874/+764) DNA construct in transfected Day 18 GE cells. Each bar represents the Least Squares Mean ±SEM (n=9) in arbitrary luciferase light units (normalized for activity of cotransfected pCAT) of triplicate plates from three separate experiments (n=3 pigs). After transfection, cells were incubated in RPMI 1640 medium containing charcoal-stripped fetal bovine serum and supplemented with vehicle (CS), 1 nM estrogen (+E2) or 25 nM progesterone (+P4) for 24 hours. The effects of estrogen (p=0.879) and progesterone (p=0.216) were not significant

Peptide Hormonal Effects on Uterine Endometrial IGFBP-2 Gene Expression

IGF-II, PMA, and cAMP increase IGFBP-2 mRNA abundance *in vitro*. Two experiments examined for possible peptide hormonal effects on IGFBP-2 gene expression. First, when primary cultures of D18GE cells reached ~ 80 % confluence, cell were placed in serum-free medium for 18 hours. In the treatment groups (three plates for each), I mM cAMP or 100 ng/ml Phorbol 12-Myristate 13-Acetate (PMA) were added. The control group (three plates) received vehicle (water). All cell cultures were then incubated for an additional 6 hours. In the second experiment, cells were treated with or without 5 ng/ml TGF-β1, 50ng/ml IGF-I or 50 ng/ml IGF-II for 16 hours. Total cellular RNA of all cell groups was subjected to RPA for IGFBP-2. Results from these experiments (Figure 5-5) demonstrated that IGF-II, PMA and cAMP all increased steady-state IGFBP-2 mRNA levels.

To confirm the stimulatory effect of PMA, D18GE cells from a second animal, were treated with three different doses of PMA (100, 200, 500 ng/ml) for 6 hours and with 200 ng/ml PMA for three different time periods (3, 6 and 9 hours). Results demonstrated that PMA effected IGFBP-2 mRNA abundance in a dose-dependent manner with linear increase from 0 to 200 ng/ml ($p<0.05$) and quadric decrease from 200 to 500 ng/ml ($p<0.6$), and that the length of treatment period used did not affect the mRNA abundance (Figure 5-6). However, cAMP and IGF-II effects on IGFBP-2 mRNA abundance were not dose-dependent (Figure 5-6).

IGF-II Effects on IGFBP-2 Promoter Activity. To attempt to localize the IGF-II response region, if present, within the 1.4 kb upstream region, the constructs Hind-LUCe,

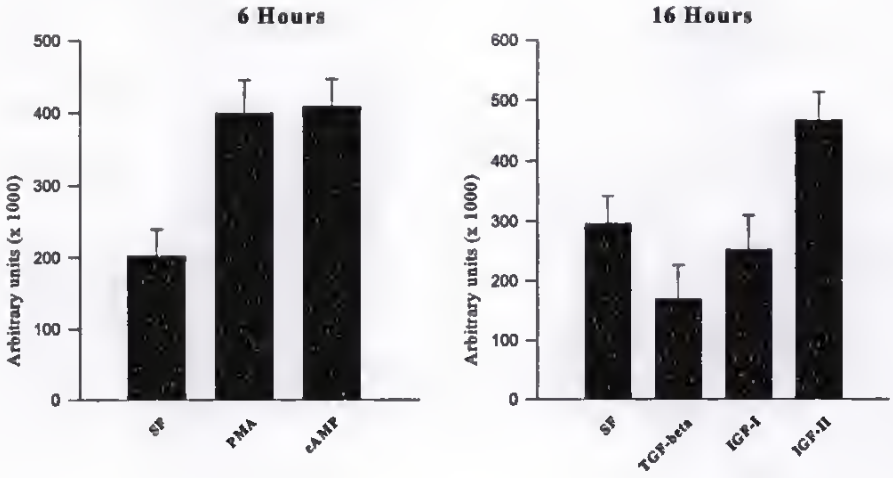


Figure 5-5. Peptide hormonal effects on IGFBP-2 steady-state mRNA abundance in endometrial glandular epithelial cells from Day 18 pregnant pigs (D18GE). Each bar represents the Least Squares Mean of phosphor imaged data from RPA. The Y-axes are arbitrary units. Cells were treated with 100 ng/ml PMA or 1 mM cAMP for 6 hours, or with 5 ng/ml TGF-β1, 50 ng/ml IGF-I, or 50 ng/ml IGF-II for 16 hours. PMA (n=3), cAMP (n=2) and IGF-II (n=3) treatments increased IGFBP-2 mRNA abundance ($p<0.05$), whereas TGF-β1 ($p=0.133$) and IGF-I ($p=0.570$) had no effect.

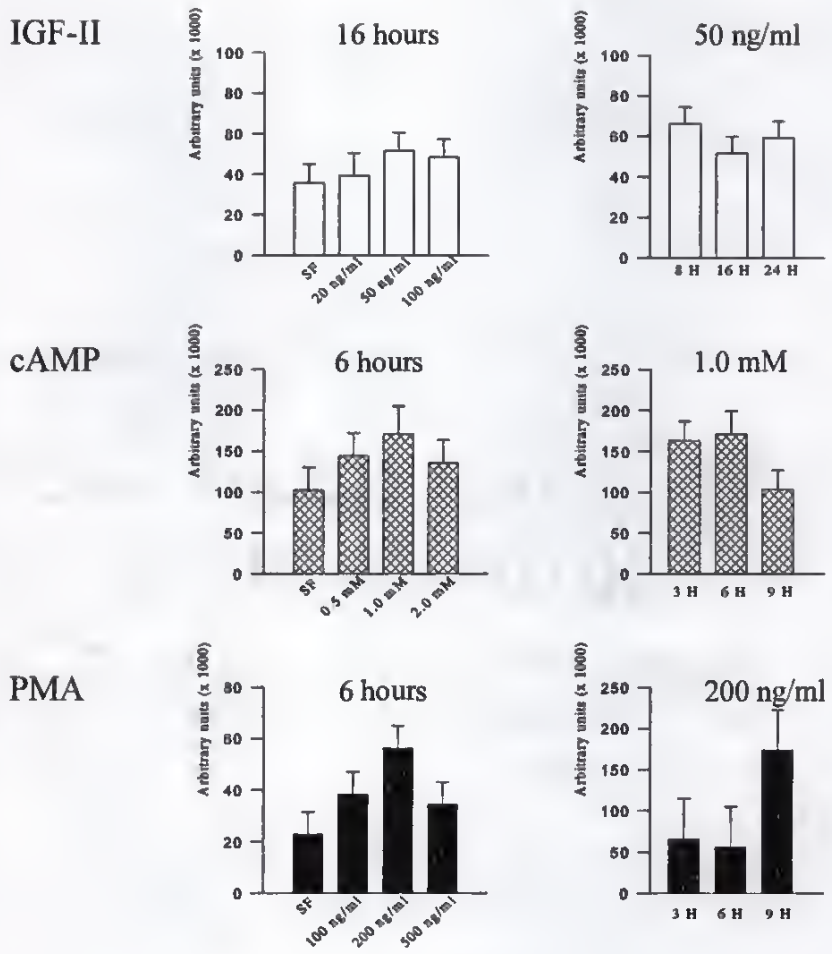


Figure 5-6. Dose-dependent effects of IGF-II, cAMP and PMA on IGFBP-2 mRNA abundance. Each bar represents the Least Squares Mean of phosphor imaged data from RPA. The Y-axes are arbitrary units. The top open bars represent the data from IGF-II treated D18GE cells. Cells were treated with 0 (SF, n=3), 20 (n=2), 50 (n=3) or 100 (=3) ng/ml IGF-II for 16 hours as shown in the left figure, or treated with 50 ng/ml IGF-II for 8 (n=3), 16 (n=3) or 24 (n=3) hours as shown in the right figure. The stippled bars represent the data from cAMP treated D18GE cells. Cells were treated with 0 (SF, n=3), 0.5 (n=3), 1.0 (n=2) or 2.0 (n=3) mM cAMP for 6 hours as shown in the left figure, or treated with 1.0 mM of cAMP for 3 (n=3), 6 (n=2) or 9 (n=3) hours as shown in the right figure. The bottom black bars represent the data (n=3 for each treatment) from PMA treated D18GE cells. Cells were treated with 0, 100, 200 or 500 ng/ml PMA for 6 hours as shown in the left figure, or treated with 200 ng/ml PMA for 3, 6 or 9 hours as shown in the right figure.

Sac-LUCe, Bgl-LUCe and Sma-LUCe were separately transfected into D18GE cells. Transfected cells were placed in RPMI 1640 medium containing 10% FBS for 32 hours. Medium was then changed to serum-free medium (SF, control group) or serum free medium supplemented with 200 ng/ml IGF-II (+IGF-II). Cells were further incubated for 16 hours. Data from these experiments (n=3 pigs) indicated no stimulatory response to IGF-II by any of the DNA constructs tested (Figure 5-7). Instead, decreases in promoter activity of all DNA constructs were observed. Although statistical support for an overall treatment effect was not obtained ($p=0.225$), IGF-II significantly decreased the promoter activity of Sac-LUCe in transfected cells ($p<0.05$).

When D18GE cells were similarly transfected with SaB(110)-pLUCe and SmB(459)-pLUC constructs, and then treated with IGF-II as described above, no effect of IGF-II was observed (Figure 5-8).

Developmental Change of IGF-II Receptor Density on Endometrial Cell Membranes

As mentioned above, IGF-II gene expression in the fetus, placenta and endometrium is temporally correlated with endometrial IGFBP-2 gene expression. In addition, IGF-II increased the IGFBP-2 mRNA abundance in D18GE cells. Therefore, IGF-II receptors on endometrial cell surfaces may be important for transducing the effects of IGF-II on the tissue- and developmental stage-specific synthesis of IGFBP-2. Ligand blot analysis using ^{125}I-labeled hIGF-II showed (Figure 5-9) that IGF-II receptor density was relatively high in cell membranes of Days 18, 30 and 60 pregnant pig endometria, and relatively low in the Days 12 and 90 endometria. Interestingly, several IGFBPs were found on the cell surface as well.

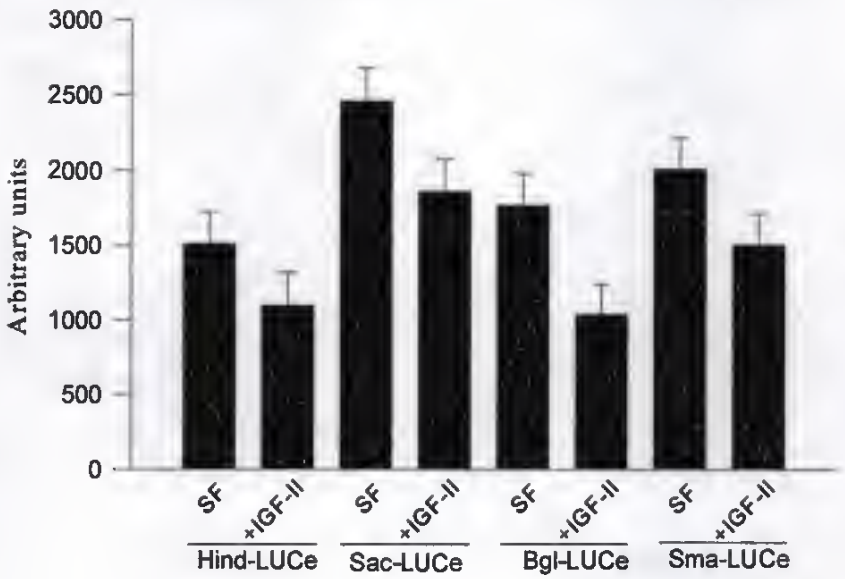


Figure 5-7. Effects of IGF-II on promoter activities of Hind-LUCe (-1397/+73), Sac-LUCe (-874/+73), Bgl-LUCe (-764/+73) and Sma-LUCe (-305/+73) DNA constructs transfected into D18GE cells. Transfected cells were treated with (+IGF-II) or without (SF) 200 ng/ml IGF-II for 16 hours. Each bar represents the least squares mean ± SEM (n=9) of arbitrary luciferase light units (normalized for cotransfected pCAT activity) from triplicate plates of three independent experiments (n=3 pigs). IGF-II decreased the promoter activity of Sac-LUCe ($p<0.05$).

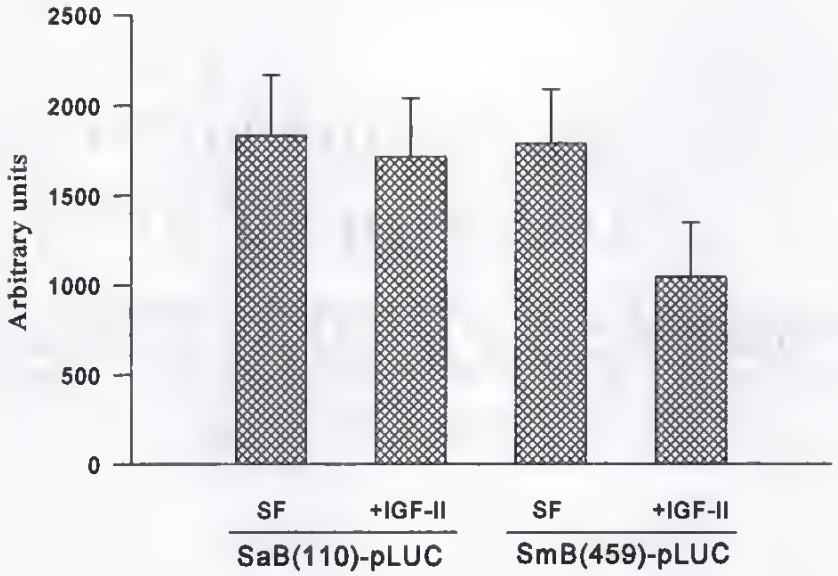


Figure 5-8. Effects of IGF-II on reporter gene activities of SaB(110)-pLUC and SmB(459)-pLUC DNA constructs transfected into D18GE cells. After transfection, cells were treated with 200 ng/ml IGF-II or without (SF) for 16 hours. Each bar represents the Least Squares Mean ± SEM (n=9) of arbitrary luciferase light units (normalized for co-transfected pCAT activity) of triplicate plates from three separate experiments (n=3 pigs). Effects of IGF-II were not significant (p=0.834 and p=0.226)

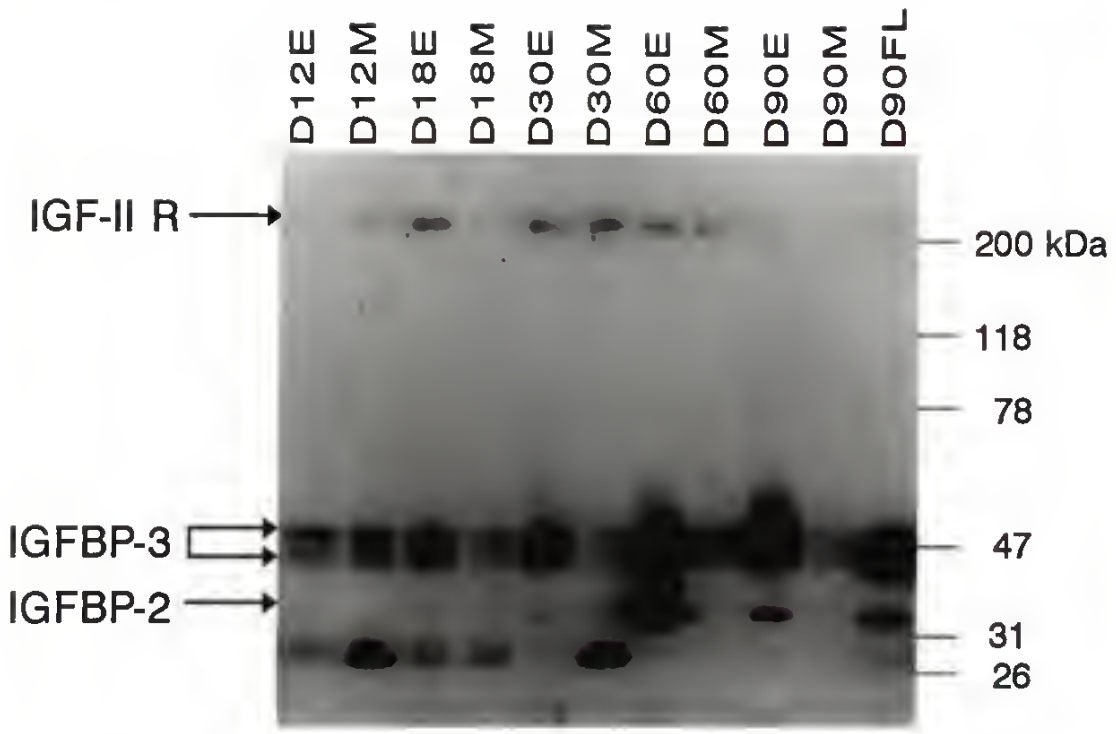


Figure 5-9. IGF-II receptor density on endometrial cell membranes. Ligand blot analysis was performed using [^{125}I]IGF-II. Equal amounts of cell membrane proteins (200 μg) from Day 12 endometrium (D12E) or myometrium (D12M), Day 18 endometrium (D18E) or myometrium (D18M), Day 30 endometrium (D30E) or myometrium (D30M), Day 60 endometrium (D60E) or myometrium (D60M), Day 90 endometrium (D90E) or myometrium (D90M), or Day 90 fetal liver (D90FL) were subject to nonreducing SDS-PAGE. Protein molecular weight markers are indicated on the right.

IGFBP-2 (34 kd) was present on cell membranes of Days 60 and 90 endometria and Day 90 fetal liver.

Differential Expression and Accumulation of IGFBP-2

IGFBP-2 mRNA steady-state abundance was higher in endometrial stromal cells than in endometrial glandular epithelial cells (D18GE) (Figure 5-10). Likewise, there was more IGFBP-2 in membranes of stromal than glandular epithelial cells (Figure 5-10).

Discussion

Effects of estrogen on IGFBP-2 gene expression have been controversial. Estrogen administration increased IGFBP-2 mRNA abundance in pituitary glands of ovariectomized rats (Michels et al., 1993). In ovarian cancer cells, however, estrogen minimally depressed IGFBP-2 mRNA level (Krywicki et al., 1993). In the human breast cancer cell line MCF-7, estrogen had no effect on IGFBP-2 gene expression (Kim et al., 1991). In previous studies in this laboratory using ovariectomized gilts treated with estrogen in a relatively low daily dosage (0.1 μg/kg body weight vs 100 μg/head in this study), estrogen effects on uterine endometrial IGFBP-2 mRNA abundance were not observed (Simmen et al., 1990). The present study provided direct evidence that estrogen stimulates IGFBP-2 mRNA abundance in both the *in vivo*-ovariectomized pig model and *in vitro* in D18GE cells. These results confirmed the basis for the close temporal correlation noted for endometrial IGFBP-2 mRNA abundance and circulating estrogen concentrations in the pig. In pregnant pigs, the concentration of estrogen (both 17β-estradiol and estrone) in peripheral serum increases throughout pregnancy (Anderson et al., 1983). Therefore estrogen is probably an important hormonal mediator of endometrial IGFBP-2 gene activity.

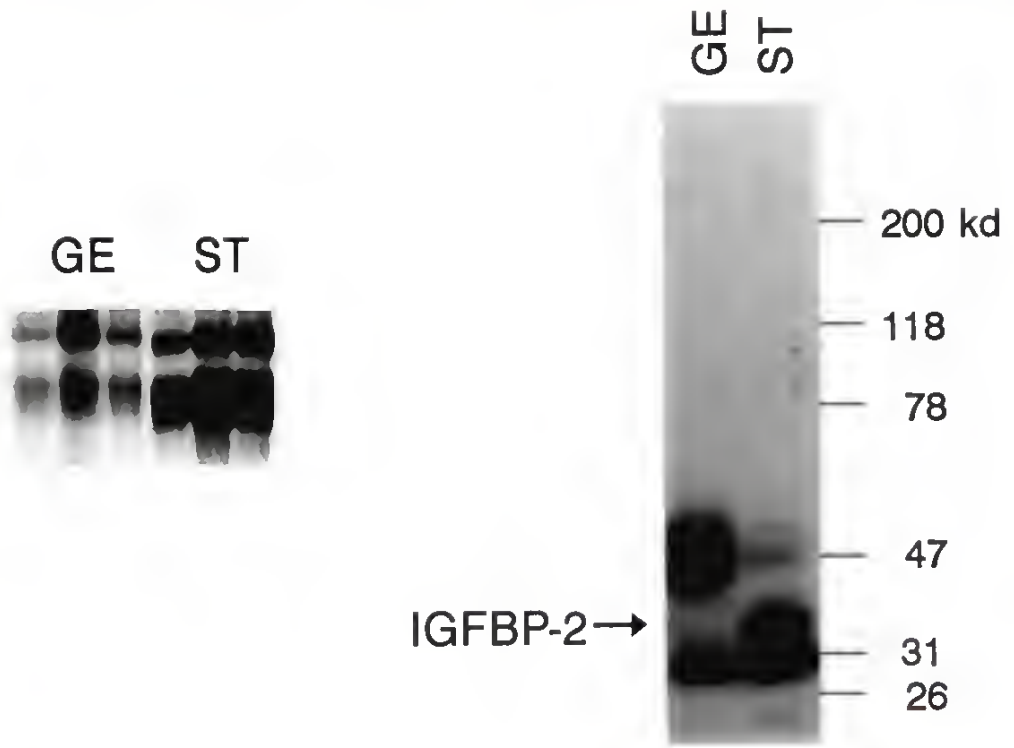


Figure 5-10. Differential expression of IGFBP-2 gene and protein in endometrial cells. Left panel, RNase Protection Assay for IGFBP-2 gene (as described in the Materials and Methods).Fifteen µg of total RNA from glandular epithelial (GE) cells of Day 18 pregnant pig endometrium, or 10 µg of total RNA from stromal (ST) cells of the same tissue was used for each reaction. Three assays for each cell type were performed. RNAs for each reaction are from separate culture plates. Right panel, ligand blot analysis using [^{125}I]IGF-II. Equal amounts of cell membrane proteins (200 µg) from GE and ST cells were subjected to nonreducing SDS-PAGE. Protein size markers are indicated on the right.

Progesterone has been shown to be a stimulator of IGFBP-2 synthesis in human endometrium (Giudice et al., 1991a, b). IGFBP-2 mRNA expression is higher in the secretory phase (under progesterone control) than the proliferatory phase (under estrogen control) (Giudice et al., 1991b). Northern blot analysis, using RNA from pooled tissues of ovariectomized gilts treated with progesterone in a relatively low daily dosage (0.2 mg/kg body weight vs 200 mg/head), showed that endometrial IGFBP-2 gene expression might be increased (Simmen et al., 1990). In pigs, the concentration of progesterone in the circulation of cyclic and pregnant pigs is negatively correlated with endometrial IGFBP-2 mRNA abundance (Simmen et al., 1992; Anderson et al., 1982). The present study using RNase protection assay showed that progesterone had stimulatory effect *in vivo*, but not *in vitro* in D18GE cells. These results may indicate that progesterone effect on IGFBP-2 mRNA abundance indirectly or no other endometrial cells, such as stroma.

Concomitant expression of IGF-II and IGFBP-2 genes has been commonly observed in previous studies. The hepatic levels of IGF-II and IGFBP-2 mRNAs are increased during the latter half of gestation and then decline postnatally (Lee et al., 1993b; Kampman et al., 1993). In rat embryo, IGF-II and IGFBP-2 gene expression is complementary in many tissues following gastrulation (Wood et al., 1992). In oxidant-arrested lung alveolar epithelial cells, the expressions of IGF-II, type II receptors and IGFBP-2 genes are induced (Cazals et al., 1994). Glucocorticoids inhibit cell proliferation of lung alveolar epithelial cells and stimulate expression of IGFBP-2, IGF-II and type II receptor genes (Mouhieddine et al., 1996). However, no direct evidence that links expression of IGF-II and IGFBP-2 has been reported to date. Results from this study showed that IGF-II stimulated IGFBP-2 gene

expression in endometrial glandular epithelial cells, and that the binding capacity of IGF-II receptors on endometrial cell surface is temporally correlated with IGFBP-2 mRNA abundance. This implies that IGF-II may have an important role in the tissue- and developmental-specific aspect of IGFBP-2 gene expression. Interestingly, during mid-pregnancy when IGFBP-2 is highly expressed in the endometrium, IGF-II in the placenta is also highly expressed, whereas IGF-II gene in endometrium is minimally expressed (Simmen et al., 1992). Moreover, maternal serum IGF-II levels decline steadily during pregnancy (Lee et al., 1993). This evidence supports the above idea and implies that fetal and placental IGF-II is a major determinant of IGFBP-2 gene expression. The fetal IGF-II paracrine regulatory scenario can be extended to late pregnancy as well. Levels of IGF-II receptors on the cell surface are decreased during late pregnancy. This decline of IGF-II receptor numbers could be a reason for the decline of endometrial IGFBP-2 mRNA abundance, which are not associated with a continued increase in fetal IGF-II gene expression during late pregnancy. Although this fetal IGF-II regulatory scenario cannot be considered as the sole model for explaining developmental and tissue specific gene expression of IGFBP-2, it does support the theory that the IGFBP-2 gene is induced at pregnancy (Simmen et al., 1992; Song et al., 1996). Collectively, these observations imply a role of the IGF system in maternal-fetal interactions (Roberts et al., 1993; Simmen et al., 1995).

IGF-II receptor is a monomeric protein and is identical to the cation-independent mannose-6-phosphate (Man-6-P) receptor. This receptor has distinct binding sites for IGF-II and Man-6-P and Man-6-P-containing glycoproteins, and can bind IGF-II and Man-6-P simultaneously (Braulke et al., 1988). Cell surface IGF-II receptors exhibit high affinity for

IGF-II, low affinity for IGF-I and no binding of insulin (Nissley et al., 1991). Although the signal transduction of the IGF-II receptor with respect to IGF-II action is controversial, it has been reported that binding of IGF-II to this receptor activates a G_{i2} protein through a 14 amino acid intracellular motif, and subsequently Ca^{++}influx and DNA synthesis are increased (Nishimoto, 1993). In the present study, PMA and cAMP had stimulatory effects on IGFBP-2 gene expression, suggesting that both PKC and PKA signal transduction pathways may subserve IGFBP-2 gene regulation. Although this signal transduction pathway remains to be investigated, it is possible that IGF-II affects the IGFBP-2 gene through PKA, PKC or both pathways.

A fetal IGF-II paracrine regulatory model does not exclude effects of other hormones. It has been reported that retinoic acid stimulates IGFBP-2 gene expression in rat hepatic cells (Schmid et al., 1992) and in bovine mammary epithelial cells (Woodward et al., 1996). Glucocorticoid (GC) rapidly decreases DNA synthesis and proliferation of lung alveolar epithelial cells, which is associated with accumulation of IGFBP-2 in the culture medium, and an increase of IGFBP-2 mRNA in these same cells (Mouhieddine et al., 1996). In the present study, IGFBP-2 mRNA and promoter activities were lower when endometrial cells were incubated in medium supplemented with 10% charcoal-stripped bovine fetal serum than in medium supplemented with 10% normal bovine fetal serum (data not shown) suggesting that lipid components in the serum, which have been removed, are important for overall IGFBP-2 gene expression. Glucocorticoids and retinoids may be candidate molecules responsible for uterine expression of IGFBP-2. Although the role of these two hormones in this regulation remains to be investigated, two lines of evidence support this hypothesis:

First, the gene encoding retinol-binding protein, the protein of which is responsible for transporting retinoids, is highly expressed in the endometrium of Days 11 to 60 of pregnant pigs and is not expressed in myometrium (Song et al., 1996; Roberts et al., 1993). This tissue-specific expression is identical to that of IGFBP-2. Second, glucocorticoid receptors recognizes the same *cis*-element as progesterone receptors (Faisst and Meyer, 1992). Likewise, the retinoic acid response element (RAR) or retinoids response element (RXR) are identical to the estrogen response element (ERE). Several half sites of RAR or RXR/ERE and GRE/PRE have been identified within the 1.4 kb 5'-upstream region of IGFBP-2 gene (data not shown). A negative correlation between IGFBP-2 and IGF-I gene expression has been observed in several other studies. In rat Leydig cells, human chorionic gonadotropin (hCG) up-regulated IGF-I (Lin et al., 1986) and IGF-I receptor gene expression (Lin et al., 1987; Nagpal et al., 1991), but decreased the gene expression of IGFBP-2 (Wang et al., 1994). In pig uterus, the IGFBP-2 gene is expessed in endometrium at low levels during early pregnancy and at high level during mid-pregnancy; while the IGF-I gene is more highly expressed in endometrium during early rather than mid-pregnancy, and more highly expressed in myometrium than endometrium (Song et al., 1996). These observations imply that IGF-I may inhibit IGFBP-2 gene expression or that IGFBP-2 inhibits IGF-I gene expression or perhaps both. The present study showed that IGF-I had no effect on IGFBP-2 gene expression. The other possibility remains to be investigated.

TGF-β1 has been proposed as a stimulator of IGFBP-2 gene expression (Cazals et al., 1994). However, in the present study, this cytokine had no effect on IGFBP-2 mRNA

steady-state abundance in endometrial cells suggesting that the effect of TGF-β1 on IGFBP-2 may be cell-type dependent.

Surprisingly, in transfection experiments, estrogen and progesterone decreased reporter gene activity in D18GE cells. Similarly IGF-II did not stimulate, but rather inhibited reporter gene activity in transfected cells. There are several possible reasons for these observations. First of all, chromatin structure may play an important role in IGFBP-2 gene expression. In the genome, gene expression is sensitive to the state of the chromatin domain, such as replication, supercoiling and loop formation (Goldman, 1988). Nucleosome formation and alteration in regulatory regions (Wolffe, 1994; Lewin, 1994), the presence of chromatin boundaries of the transcription units (Levy-Wilson, 1995), and the nuclear matrix-DNA interactions (Goldman, 1988) are all considered to be important aspects of transcriptional regulation. The present study used transient transfection of reporter genes, which is commonly used for determination of interactions of cis- and trans-elements. However, this system does not reflect the native conditions of gene regulation. Secondly, the major cis-element(s), mediating the stimulatory effects of estrogen and IGF-II, may be located in further upstream and/or downstream locations than the region studied, whereas a minor silencing region, which directly or indirectly responds to estrogen and IGF-II, is located within the region from -764 to +73. In the case of a transient transfection system, the major response region may be excluded. The effect of the minor silencing response region therefore becomes more apparent. Thirdly, IGFBP-2 has an RGD sequence on the C-terminus of the peptide (Song et al., 1996). The RGD sequence is a common motif found in a variety of extracellular adhesion proteins, and is recognized by αβ integrin receptors -- a

family of homologous cell surface receptors (Hynes et al., 1992; Jones et al., 1993; Delhanty and Han 1993b). It is therefore possible that IGFBP-2 may have an auto-regulatory function via the RGD sequence and αβ integrin receptors. The observations that IGFBP-2 is present on endometrial cell membranes (this Chapter) and that IGFBP-2 directly interacts with cell membranes in another study (Russo et al., 1996), support this model. In the transfection system, any endogenously synthesized IGFBP-2 as stimulated by estrogen or IGF-II, would be secreted and accumulate in the medium. This IGFBP-2 could bind integrin receptors or other cell membrane proteins on the cells and exert potential negative feedback on IGFBP-2 transcriptional activity. The potential negative response region for this postulated feedback is located in region from -764 to +73. Such a model could explain why the mRNA level for IGFBP-2, when endometrial cells were treated with IGF-II and estrogen, was increased whereas the reporter gene activity was decreased. A speculative model incorporating these results is presented in Figure 5-11. Nevertheless, the exact mechanism underlying these differential effects remains to be investigated.

It has been reported that IGFBP-2 is expressed in both endometrial stromal and epithelial cells (Simmen et al., 1992). RNase protection assays showed that the mRNA abundance of IGFBP-2 is higher in primary cultures of endometrial stromal cells than glandular epithelial cells. These data indicate that IGFBP-2 is mainly synthesized in stromal cells. Ligand blot analysis showed more IGFBP-2 on cell membranes of stroma than epithelia, consistent with the cell culture studies . However, immunohistochemical localization showed that IGFBP-2 mainly accumulates on cell surfaces of luminal and glandular epithelium (Song et al., 1996). This observation may imply that the uterine stromal

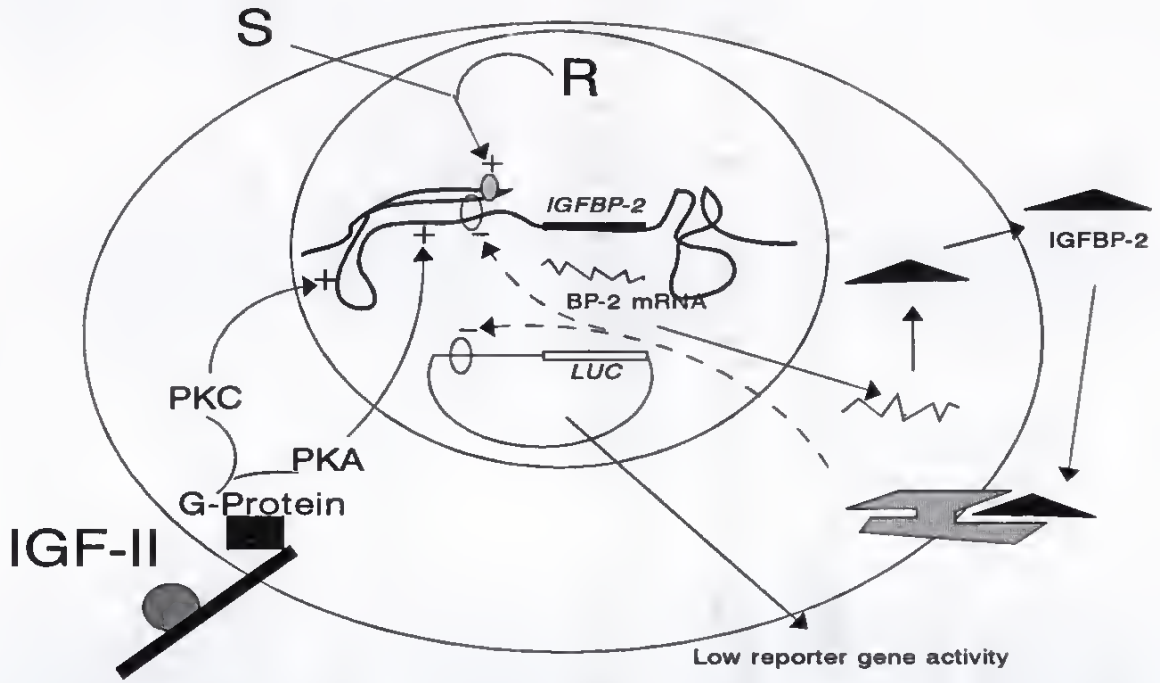


Figure 5-11. Speculative model for trophic regulation of the IGFBP-2 gene.

synthesized IGFBP-2 is transported to the surface of uterus-the boundary between fetal and maternal tissues. Although it remains to be investigated, functions of this accumulated IGFBP-2 can be speculated to be the following: First, IGFBP-2 may protect the uterine tissue by inhibiting the effect of fetal IGF-II on endometrial cell proliferation. During mid- and late-gestation fetal tissues synthesize large amounts of IGF-II for stimulation of fetal growth. However, this stimulatory effect may not be required for uterine tissues at this stage. IGFBP-2 at the boundary may inhibit those IGF-II effects. It has been reported that IGFBP-2 inhibits the stimulatory effect of IGF-II on DNA synthesis and that IGFBP-2 alone inhibits DNA synthesis in the NSCLC cell line (Reeve et al., 1993). Second, IGFBP-2 may be a unique extracellular matrix associated protein linking uterine and placental tissues through its RGD or other sequence motifs. Further studies on this protein's functions in utero will be helpful for resolving these issues.

Summary

The IGFBP-2 gene exhibits a relatively unique tissue- and developmental stage-specific expression pattern in pig endometrium. However, there is a general lack of knowledge concerning this gene's regulation. Results of the present study showed that:

I). Estrogen stimulates IGFBP-2 gene expression at the level of mRNA in the *in vivo*-ovariectomized pig model and *in vitro* using D18GE cells. Progesterone stimulatory effect were observed in the *in vivo*-ovariectomized pig, but *in vitro* using D18GE cells.

2). IGF-II, PMA and cAMP elicited increases in IGFBP-2 mRNA abundance in primary cultures of endometrial glandular epithelial cells.

3). The binding capacity and/or number of IGF-II receptors on endometrial cells was correlated with maximal gene expression of IGFBP-2 during pregnancy. Therefore fetal and placental IGF-II is a candidate hormonal regulator of IGFBP-2 gene expression.

4). No estrogen or IGF-II stimulatory response regions were identified in the 1.4 kb upstream region of the IGFBP-2 gene, using the transient transfection approach. Instead, a region, which mediated the negative effects of estrogen, progesterone and IGF-II on IGFBP-2 gene promoter activity was identified within -764 to +73 nucleotides relative to the ATG translational initiation codon.

CHAPTER 6
DNA METHYLATION OF THE CpG ISLAND ENCOMPASSING THE PROMOTER OF THE PIG IGFBP-2 GENE AND ITS EFFECT ON TRANSCRIPTIONAL INITIATION

Introduction

DNA methylation in mammalian cells occurs at the 5th position of cytosine (C). It is generally accepted that most 5-methylcytosines in DNA are found in the dinucleotide sequence CpG (Bird,1993). In the genome, CpG dinucleotide sequences are unevenly distributed. In selected regions, so called CpG islands, the density of CpG dinucleotide sequences is higher than the average frequency for the entire genome. Interestingly, the 5'-ends of many housekeeping as well as tissue-specific genes overlap with CpG islands. A number of studies have shown such that genes tend to be undermethylated in the tissues where expressed and methylated in the tissues where not expressed (reviewed by Razin and Kafri, 1994). The silenced genes can be activated by treatment of cells with the demethylating agent, 5-azacytidine (Ferguson et al., 1995). The active genes can be silenced by DNA methylation (Herman et al., 1994). Therefore, DNA methylation has been implicated in the control of mammalian gene expression.

The G/C rich promoter and exon 1 of the IGFBP-2 gene has been conserved across species (Binkert et al., 1992; Boisclair et al. 1993). In the pig, this gene is expressed in a tissue and developmental-specific manner (Simmen et al., 1992). Whether DNA methylation

of this gene differs in expressing vs. nonexpressing tissues and whether this gene is controlled at the level of transcription by DNA methylation are potentially important questions. However, data concerning the relative DNA methylation state of the lGFBP-2 gene from any species has not been reported to date. In this chapter, a common method, digestion with methylation-sensitive enzymes, was used to examine the DNA methylation status of the IGFBP-2 gene promoter and exon 1 region in a highly expressing tissue (endometrium of Day 30 pregnant pigs) and in low or no expressing tissues (uterine myometrium and placenta of Day 30 pregnant pigs). Furthermore the effect of DNA methylation on activity of the lGFBP-2 gene promoter was examined using a standard cell-free *in vitro* transcription system.

Materials and Methods

DNA Sequence Analysis:

DNA sequences were compiled and analyzed using the Sequence Analysis Software Package of the Genetics Computer Group, Inc. (GCG Package, Version 7.0, 1991). Percentage G/C content was determined by use of the "window" command with a 100 bp window size and a 1 bp increment.

Genomic Southern Blot Analysis:

Genomic DNAs from endometrium, myometrium and placenta of Day 30 pregnant pigs were prepared as previously described (Ausubel et al., 1987). Briefly, 1 g of tissue was incubated in 12 ml of digestion buffer (100 mM NaCl, 10 mM Tris-Cl, pH 8.0, 25 mM EDTA, pH 8.0, 0.5 % sodium dodecyl sulfate, 0.1 mg/ml proteinase K) at 50 °C for 18 hours with shaking. Samples were extracted with an equal volume of phenol/chloroform/isoamyl

alcohol (25:24:1), and centrifuged at 10,000 rpm for 10 min to separate phases. DNA in the supernatant was precipitated in ethanol and resuspended in TE buffer.

For genomic Southern blots, 75 μg of genomic DNA from each tissue was digested with 50 units of 5-methylcytosine-sensitive restriction endonuclease (*Not* I, *Ksp* 1, *Sma* I, or *Ecl* XI) overnight. Additional enzyme was added and the sample incubated for a total of 48 hours. DNAs were precipitated and resuspended in water. All samples, including the control (*Hin*d 1II only), were digested with 80 units of *Hin*d III for 24 hours and then subjected to standard Southern blot procedures. Hybridization to random primer-labeled probes was performanced at 55 °C for an overnight period.

In vitro Methylation and Transcription:

A DNA fragment (from positions -305 to +73 relative to the translational initiation codon) was isolated from the plasmid #81 by cleavage with *Sma*l and *Hin*dIII. This fragment was methylated *in vitro* using *Sss*1 CpG Methylase (New England Biolabs, Beverly, MA) in 50 mM NaCl, 10 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM DTT, pH 7.9, with 160 or 320 μM S-adenosylmethionine and incubation at 37 °C for 1.5 hours or overnight. One or two hundred ng of the methylated DNA fragment was used as template for *in vitro* transcription using the Hela Cell Extract System according to manufacturer's specifications (Promega, Corp., Madison, WI).

Results

GC Content of IGFBP-2 Gene

All four IGFBP-2 gene exons were found to be very G/C rich (Figure 6-1). Exon 1 and its immediate 5' flanking region were extremely high in G/C content (>70%), and this

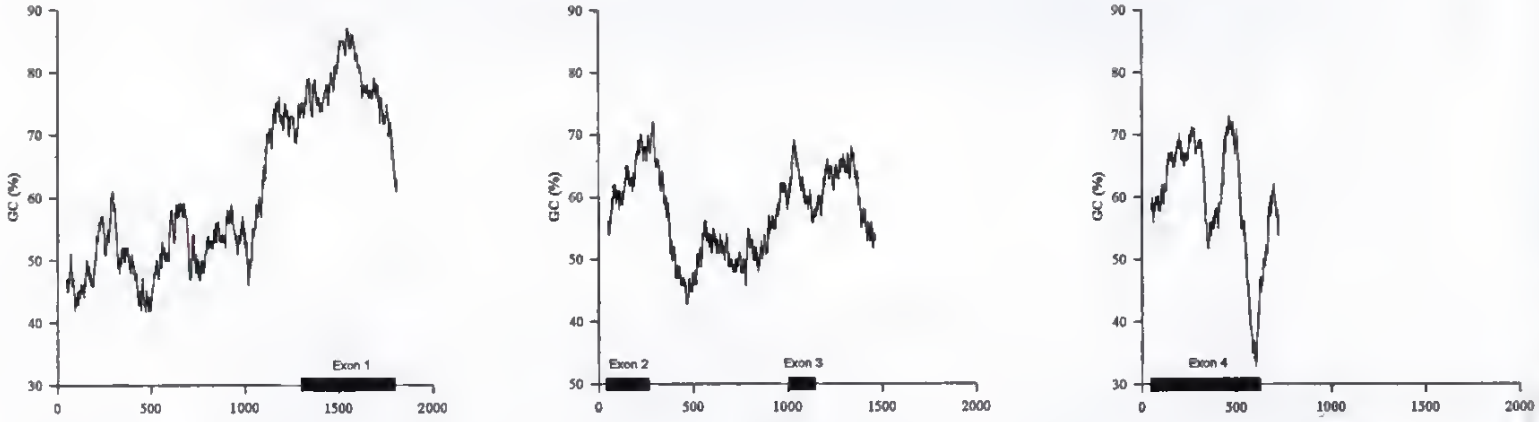


Figure 6-1. All exons of the IGFBP-2 chromosomal gene are G/C rich and exon 1 and immediate 5' flanking region constitutes a CpG island. The percentage G/C content (Materials and Methods) is plotted relative to the DNA sequence (Chapter 3) of each exon of the IGFBP-2 gene. The ratio of CpG/GpC in 5' flanking region and exons 1 (-305 to +464) is 0.73, while those in exons 2, 3 and 4 are 0.5, 0.4 and 0.44, respectively.

region exhibited hallmarks of a CpG island (Figure 6-1). In particular, there was a high frequency of CpG doublets (11%) and a cluster of recognition sites for rare-cutting restriction endonucleases, such as *Bss*HII, *Nae*I, *Nar*I, *Not*I and *Sma*I (data not shown)

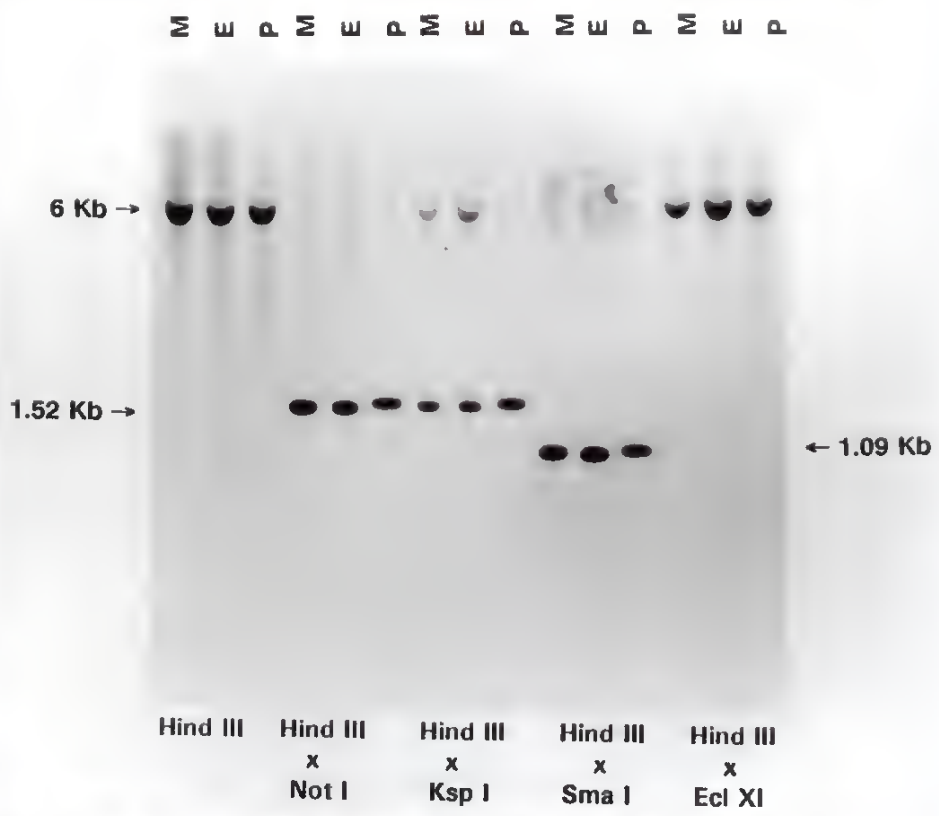
DNA Methylation of Exon 1 and Promoter of the IGFBP-2 Gene

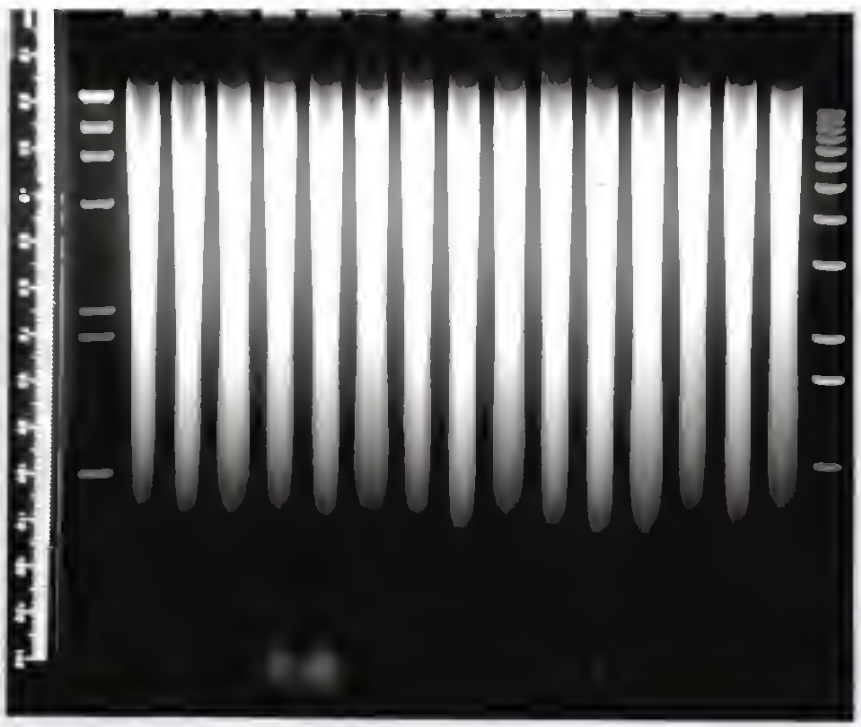
In order to examine the DNA methylation status in the promoter region of the IGFBP-2 gene, a genomic Southern blot experiment was designed (Figure 6-3). Genomic DNA from endometrium, myometrium and placenta of a Day 30 pregnant pig was digested with *Hin*d III or with *Hin*d III and a 5-methylcytosine sensitive restriction enzyme. Results presented in Fig. 6-2 showed no DNA methylation differences between the high IGFBP-2 expressing tissue (endometrium) and the low IGFBP-2 expressing tissues (myometrium and placenta) at the sites recognized by these enzymes, suggesting that DNA methylation at these sites is not important for IGFBP-2 gene activity.

Lack of Effect of DNA Methylation on IGFBP-2 Promoter Activity *In Vitro*

To confirm that differential DNA methylation may not be important for transcriptional regulation of the IGFBP-2 gene, an *in vitro* transcription system using Hela cell extract was employed. In the first experiment, a plasmid fragment spanning from nucleotides -305 to +73, was methylated *in vitro* in a buffer containing 160 μM S-adenosylmethionine at 37 °C for 1.5 hours. When the methylase-treated DNA was digested with *Hpa* II, about 50 % of the DNA was not cleaved and therefore was not methylated (Figure 6-4). Equal amounts (100 ng) of unmethylated or methylase-treated DNA were used as templates for the *in vitro* transcription system. No differences in transcriptional efficiencies

Figure 6-2. Genomic Southern Blot Assay of methylated IGFBP-2 DNA. Chromosomal DNA (75 μg) from myometrium (M), endometrium (E) and placenta (P) of a Day 30 pregnant pig was digested with *Hin*dIII, *Hin*dIII plus *Not* I, *Hin*dIII plus *Ksp*I or *Hin*dIII plus *Ecl*XI respectively, and electrophoresed in a 0.8 % agarose gel (top). The DNA in the gel was denatured and transferred to a nylon membrane which was hybridized with a radiolabeled IGFBP-2 genomic DNA fragment (from nucleotides -1379 to -765). Top: Autoradiography. Bottom: ethidium bromide staining of the gel prior to transfer of DNA.


M E P M E P M E P M E P M E P
6 Kb →
1.52 Kb →
← 1.09 Kb
Hind III
Hind III x Not I
Hind III x Ksp I
Hind III x Sma I
Hind III x Ecl XI



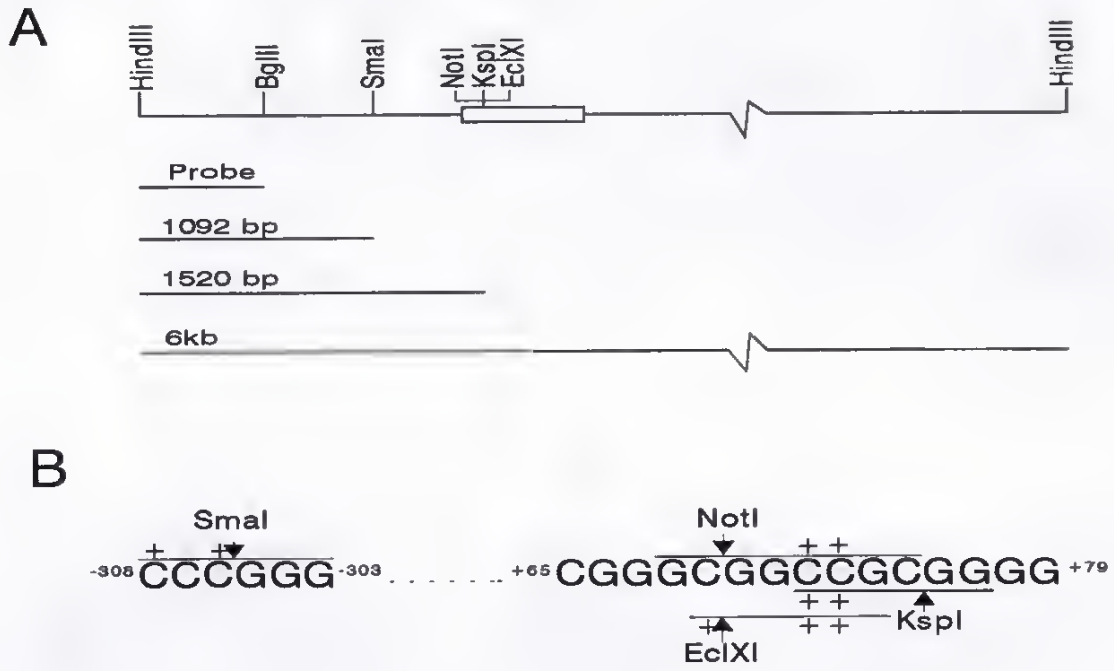


Figure 6-3. Schematic summary of the results of Southen blot analysis. Nucleotides, of which methylation inhibits respective enzyme cleavage, are labeled with a "+".

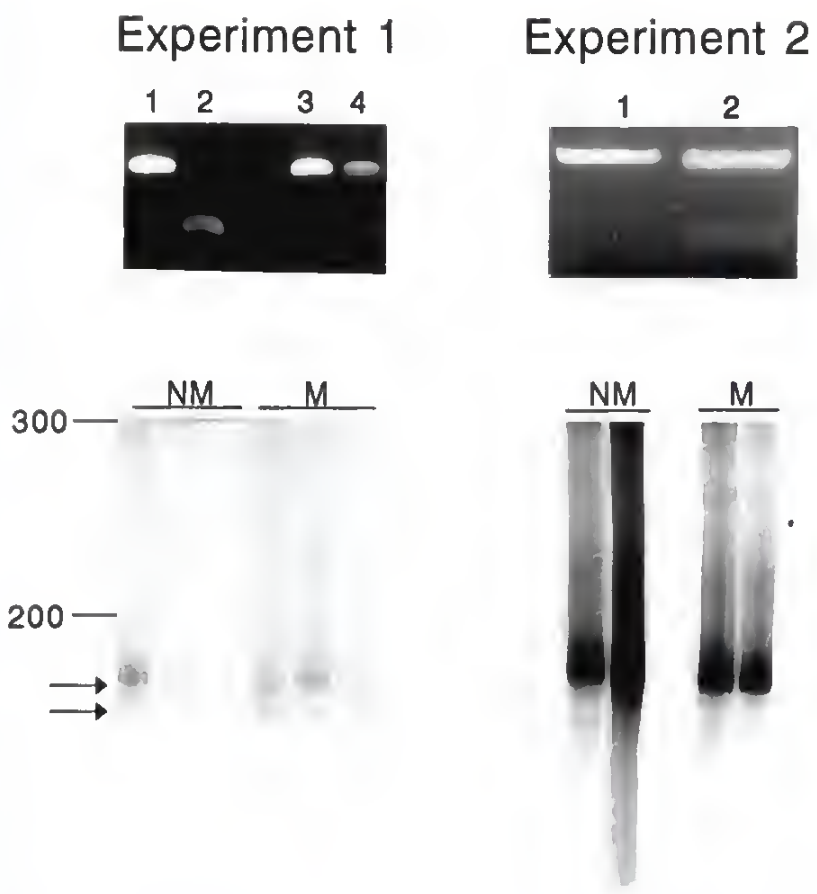


Figure 6-4. *In vitro* activity of a methylated promoter of the IGFBP-2 gene. In experiment 1, the top panel shows methylation status: lane 1 is DNA that was not methylase-treated; lane 2 is untreated DNA cleaved with *Hpa* II; lane 3 is methylase-treated DNA; lane 4 is methylase-treated DNA cleaved with *Hpa* II. The bottom panel shows the *in vitro* transcripts (triplicate lanes) from 100 ng of untreated templates (NM) or 100 ng of methylase-treated templates (M). In experiment 2, methylase-treated DNA (lane 1) was digested with *Hpa* II (lane 2). Bottom panel shows the transcripts (duplicate lanes) from 200 ng of unmethylated templates (NM) or 200 ng of methylase-treated templates (M). The expected sizes of the run-off transcripts are from 167 to 176 and from 185 to 189 nucleotides. The arrows identify the two clusters of transcriptional start sites as previously described in Chapter 3 .

were observed between the two templates suggesting that DNA methylation of this region does not affect transcriptional initiation and/or elongation *in vitro* (Figure 6-4). In the second experiment, the fragment spanning from nucleotides -305 to +73 was methylated in the same buffer only containing 320 μM S-adenosylmethionine at 37 °C for 16 hours. Analysis with *Hpa* II indicated that about 90 % of the DNA was now methylated (Figure 6-4). When 200 ng of control or methylated DNA fragment were added to the *in vitro* transcription system, transcriptional differences between the two templates were not observed (Figure 6-4).

Discussion

CpG islands are characterized by high (> 50%) G/C content and the occurrence of CpG dinucleotide pairs at greater than the average frequency (CpG /GpC ratio greater than 0.6). CpG islands are usually identified by a cluster of sites for two or more rare-cutting restriction enzymes (Larsen et al., 1992). In the case of the IGFBP-2 CpG island encompassing exon 1, there are multiple sites for a number of such enzymes and the G/C content is extremely high (maximum of 90%). CpG islands are associated with the 5' ends of many genes, the great majority of which are cellular housekeeping (Dynan, 1989; Gardiner-Garden and Frommer, 1987). However, a number of growth control genes (i.e. those encoding protooncogenes, growth factors, growth factor receptors) are also characterized by the presence of one or more CpG islands near to or overlapping their promoters (Gardiner-Garden and Frommer, 1987; Dynan, 1989); the IGFBP-2 gene would appear to fall in this class. This gene is somewhat tissue-restricted in its expression and as demonstrated in previous studies, is developmentally regulated in fetal hepatic and maternal endometrial and mammary tissues during pregnancy (Lee et al., 1993a, b; Chapter 3). It is

interesting to note that most of the known tissue-specific genes that have CpG islands at or near their 5' ends also exhibit the canonical TATA transcriptional initiation motif. The IGFBP-2 gene, in contrast, does not manifest this sequence again consistent with other genes involved in control of cellular growth (Dynan, 1989).

The promoter of the IGFBP-2 gene contains four highly conserved G/C boxes (Binkert et al., 1992; and Chapter 3), which bind to transcription factor SP1 (Boisclair et al., 1993). It has been reported that methylation of the SP1 binding element has no effect on the factor's binding (Harrington et al. 1988; Holler et al 1988) or on activation of transcription *in vivo* or *in vitro* (Holler et al., 1988). Sp1 elements also protect CpG islands from *de novo* methylation (Brandeis et al., 1994). Most housekeeping gene have CpG islands which are constitutively unmethylated (Bird, 1986; Razin and Kafri, 1994). It is possible therefore that binding of Sp1 or other members of the GC box binding protein family of transcription factors results in methylation-free CpG islands.

Restriction enzymes, which are sensitive to the presence of 5-methylcytosine within their recognition sites, such as *Not* I and *Sma* I, are commonly used for methylation studies. In the present study, three CpG dinucleotides were examined by use of 4 different restriction enzymes. Two of these (-306 and +73) were found to be unmethylated, whereas the third (+69) was methylated. Nonetheless, no differences were found with respect to degree of methylation at these three sites between the tissues studied, suggesting that DNA methylation is not the major factor for tissue-specific gene regulation of IGFBP-2.

Methylation sensitive-restriction enzyme mapping has traditionally been used to study DNA methylation. The advantage of this method is that the methylation state can be easily

examined in relatively large regions. However, regions to be studied are restricted to the relevant enzyme recognition sites. Recently, an improved genomic sequencing method was reported (Frommer et al., 1992 ; Hornstra and Yang, 1993), that allows for the determination of methylation state at each individual cytosine. A previous methylation study conducted using high-resolution genomic sequencing showed that within the same CpG island, methylation status of each CpG dinucleotide can differ (Hornstra and Yang, 1994). The observation of two unmethylated sites and one methylated site in the present study may indicate positional differences in methylation state of this gene. Therefore, the methylation status of other CpG dinucleotides present within the 5'-CpG island of the IGFBP-2 gene remains to be examined.

Coupling of *in vitro* DNA methylation and *in vitro* transcription has been used previously (Sanae et al., 1989). In the present study, template DNAs with different degree of methylation were used for *in vitro* transcription. No differences were noted between treatment and the control reactions suggesting that DNA methylation has no effect on this promoter activity *in vitro* in HeLa cell free extracts.

Summary

This study demonstrated the following:

1). All four IGFBP-2 gene exon sequences are GC-rich. The exon 1 and flanking promoter regions are embedded in a CpG island.

2). No differential methylation between high and low IGFBP-2 expressing tissues was observed by limited analysis of the IGFBP-2 promoter and exon-1 region.

3). DNA methylation did not affect IGFBP-2 promoter activity when tested in an *in vitro* transcription system derived from Hela cells.

In conclusion, although the IGFBP-2 gene is very GC rich, DNA methylation may not constitute a major factor underlying transcriptional regulation of this gene *in utero*.

CHAPTER 7
EXPRESSION OF RECOMBINANT PORCINE INSULIN-LIKE GROWTH FACTOR-BINDING PROTEIN-2 (rpIGFBP-2)

Introduction

The insulin-like growth factors, IGF-I and IGF-II, are polypeptide growth factors exhibiting structural and sequence relatedness to proinsulin. These two growth factors have insulin-like activity as well as multiple functions with respect to cell growth and development including, inhibition of apoptosis and stimulation of cell proliferation, differentiation (reviewed by Jones and Clemmons, 1995). The biological actions of IGFs are modulated, in part, by interaction with their binding proteins (IGFBPs), a family comprised of six or more members. Although a common feature of these proteins are their high affinity of IGF, each of these proteins has distinct IGF-associated and perhaps IGF-independent functions by virtue of their differential expression and individual post-translational modifications (Clemmons, 1993).

IGFBP-2 was first purified from the conditioned media of BRL-3A and MDBK cell lines and accordingly designated BRL-3A- derived IGFBP and MDBK -derived IGFBP (Drop et al., 1992). Purified IGFBP-2 from these cell lines has a higher affinity for IGF-II than for IGF-I and primarily inhibits IGF actions at the cellular level (Ross et al., 1989; McCusker et al., 1991). Recombinant human IGFBP-2 containing an extra cysteine at the C-

terminus had an inhibitory effect on IGF-I stimulated cell proliferation and protein synthesis (Feyen et al., 1991). A more recent study demonstrated that mutation of the C-terminal cysteines of this protein result in different binding affinities for IGFs (Coulter et al., 1995), suggesting that the 18 conserved cysteines residues in IGFBPs are important for their native structural conformation. In vitro studies have demonstrate that IGFBP-2 binds to cell membrane proteoglycans through its glycosaminoglycan binding site (PKKLRP) (Russo et al., 1996). The IGFBP-2 gene has been cloned and sequenced from several species (Binkert et al., 1989; Brown et al., 1989; Margot et al., 1989; Brown and Rechler, 1990; Bourner et al., 1992; Delhanty and Han, 1992; Kutoh et al., 1993; Landwehr et al., 1993; Schoen et al., 1995). In the pig uterus, steady-state abundance of IGFBP-2 mRNA is induced during periimplantation to reach maximal levels at mid-pregnancy, whereas little or no expression occurs in myometrium or placenta (Simmen et al., 1992; Song et al., 1996). This unique expression pattern implicates a physiological role for IGFBP-2 in implantation and subsequent fetal-maternal interactions. However, this issue has not been addressed, to date. Previous studies have shown that IGFBP-2 is associated with membranes of endometrial cells of pregnancy (Chapter 5). How this protein might interact with and affect uterine cellular functions, in the presence and absence of IGF ligand, is therefore an interesting question. As a prerequisite to conduct such functional studies, recombinant porcine lGFBP-2 (rpIGFBP-2) was expressed and purified from E. coli cells, and initially characterized as to its functional binding of IGF-I. Results are described in this Chapter.

Materials and Methods

Full-Length IGFBP-2 cDNA Clone

A pig IGFBP-2 cDNA clone, in the pBluescript SK(-)vector and flanked by Eco RI (5'-end) and Xho I (3'-end) sites, was obtained from the laboratory of Dr. D.R.Clemmons, Department of Medicine, University of North Carolina at Chapel Hill. The 5' and 3' ends of this cDNA were resequenced using the T3 primer (for the 5'-end) and T7 primer (for the 3'-end). DNA sequencing was performed as described in Chapter 3.

PCR Amplification of Coding Sequences for Mature IGFBP-2

A cDNA fragment encoding the mature form of IGFBP-2 was amplified by PCR from the pig cDNA fragment previously obtained by cleavage with *Eco* RI and *Xho*I. A 5' primer (Exp-DN: 5'-AGCCATATGGAGGTGCTGTTCCGCTGCCCG-3') was designed in which an *Nde* I recognition site (underlined) was incorporated. A 3' primer (Exp-UP: 5'-TTGCTCAGCCTGCATCCGCTGGGTGTGTGC-3') was designed in which a *Blp* I recognition site (underlined) was incorporated. The PCR mixture contained 50 ng of template, 200 ng of each primer, 1X buffer C (The PCR Optimizer™ Kit, Invitrogen Co, San Diego, CA), 250 μM dNTPs and 10 % DMSO. Cycling parameters were as follows:

1. Preheat	94 °C for 2 min;
2. Hot start	80°C for addition of 1 unit Expand™ High Fidelity Polymerase (Boehringer Mannheim, Indianapolis, IN);
3. Denaturation	94 °C for 30 seconds;
4. Annealing	69 °C for 40 seconds with 0.3 °C decrement/cycle;
5. Extension	72 °C for 3 min;

6. Repeat steps 3 to 5 for 10 cycles;

7. Denaturation 94 °C for 30 seconds;

8. Annealing 66 °C for 40 seconds;

9. Extension 72 °C for 3 min with 20 seconds incremental increase/cycle;

10. Repeat steps 7 to 9 for 20 cycles;

11. Extension 72 °C for 10 min.

PCR products were ligated in pCRT™ II vector (TA cloning Kit; Invitrogen Co.) and used in transformation of BL21(DE3) strain of E. coli cells. Three clones were obtained and designated BP2-47-2, -3 and -6.

Generation of IGFBP-2 Bacterial Expression Construct

The plasmid DNAs from the clones BP2-47-2, -3 and -6 were cleaved with *Blp* I + *Nde* I. The cDNA fragment encoding mature IGFBP-2 was isolated from each clone by agarose gel electrophoresis onto DEAE paper, and were separately ligated to the pET-15b vector at its unique *Nde* I and *Blp* I sites. The three constructs were first amplified in JM109 cells, and the resultant clones designated BP2-48-1, -49-1, and -50-1, respectively. All three plasmids were sequenced and were identical to wild type.

Expression and Purification of Recombinant Porcine IGFBP-2 Protein

Plasmids BP2-48-1, -49-1 and -50-1 were separately used to transform the BL21(DE3) strain of E.coli cells (Novagen, Inc., Madison, WI), and the three resultant clones designated BP2exp-48, BP2exp-49 and BP2exp-50, respectively. These bacterial cells were grown to an OD_{600} of 0.6 in LB medium containing 100 μg/ml ampicillin. IPTG was then added to a final concentration of 100 mM to induce the synthesis of IGFBP-2. After this

onset of induction, cells were incubated for an additional 2 hours, and were then collected by centrifugation at 4500 rpm for 5 min. Cell pellets were resuspended in binding buffer (5 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9), and sonicated on ice using 5 bursts (2 to 3 seconds each). Sonicates were centrifuged at 20,000 rpm for 30 min. The cell supernatant and pellets (inclusion bodies) were separately collected for column chromatography. The recombinant IGFBP-2 fused to a histidine tag was purified by affinity column chromatography on His·Bind resin (Novagen, Inc., Madison, WI).

Ligand Blot Analysis

The procedure was described previously by Mathieu et al. (1990). Briefly, proteins was boiled for 3 min in gel sample buffer (omitting 2-mercaptoethanol) and subjected to 7.5% SDS-PAGE under nonreducing conditions. Proteins were electroblotted from the gel to a nitrocellulose membrane. The membrane was first incubated in 10 mM Tris, 0.9% NaCl, 0.1% Tween 20, 0.3% Triton X-100, 0.2% BSA, 1% nonfat dry milk, and 0.1% NaN_3 for 24 h at 4 °C and then placed in binding buffer (25 mM Tris, pH 7.4, and 10 mM $MgCl_2$) containing [^{125}I]IGF-II (80,000 cpm/ml) for 3 hours at room temperature. The membrane was washed in 20 mM Tris, pH 7.4, containing 10 mM $MgCl_2$, and 0.1% Tween 20 (2x15 min washes) and then in 20 mM Tris, pH 7.4, containing 10 mM $MgCl_2$, for 20 min.

Results

Generation of an Expression Construct for Recombinant Porcine IGFBP-2

The nearly full-length cDNA clone obtained from the laboratory of Dr. David Clemmons, Department of Medicine, University of North Carolina at Chapel Hill, was sequenced using a T3 primer (for the 5'-end) and T7 primer (for the 3'-end). Sequencing data

from both laboratories showed that the genomic sequences (Chapter 3) and the cDNA sequences are identical, except for a single bp difference (C in cDNA; A in genomic DNA) in the 5'- noncoding region and 3 bp (ACC) missing in the 3'-noncoding region of the cDNA (Figure 7-1).

The original cDNA fragment was isolated (*Eco*RI + *Xho*l cleavage) and used as template to amplify the coding sequences for mature lGFBP-2 via PCR. Unique *Nde* I and *Blp* I sites were incorporated in the 5'- and 3'-primers to be used for the PCR. Interestingly, when DMSO was added in the PCR reaction, a product of expected size (0.9 kb) was obtained, whereas no product was obtained when DMSO was omitted (Figure 7-2).

The PCR product with modified ends and containing the coding sequence for mature lGFBP-2 was cloned into the pCRT II vector. Three clones were obtained and designated BP2-47-2, -3 and -6. These three clones were separately digested with *Nde* I and *Blp* I. For all three plasmids, the insert between the *Nde* I and *Blp* I sites was isolated and inserted into the pET 15b vector at its unique *Nde* 1 and *Blp* I sites. These plasmids were first amplified in E. coli JM 109 cells. Three corresponding clones were obtained and designated BP2-48-1 (BP2-47-2 insert), BP2-49-1 (BP2-47-3 insert) and BP2-50-1 (BP2-47-6 insert). Sequencing showed all three plasmid inserts to have identical sequences as the original cDNA indicating that no mutations were introduced during PCR. This construct was predicted to express the full-length mature IGFBP-2 protein also containing 4 additional amino acids at the N-terminus (after cleavage with thrombin) and 3 additional amino acids at the C-terminus (Figure 7-3).

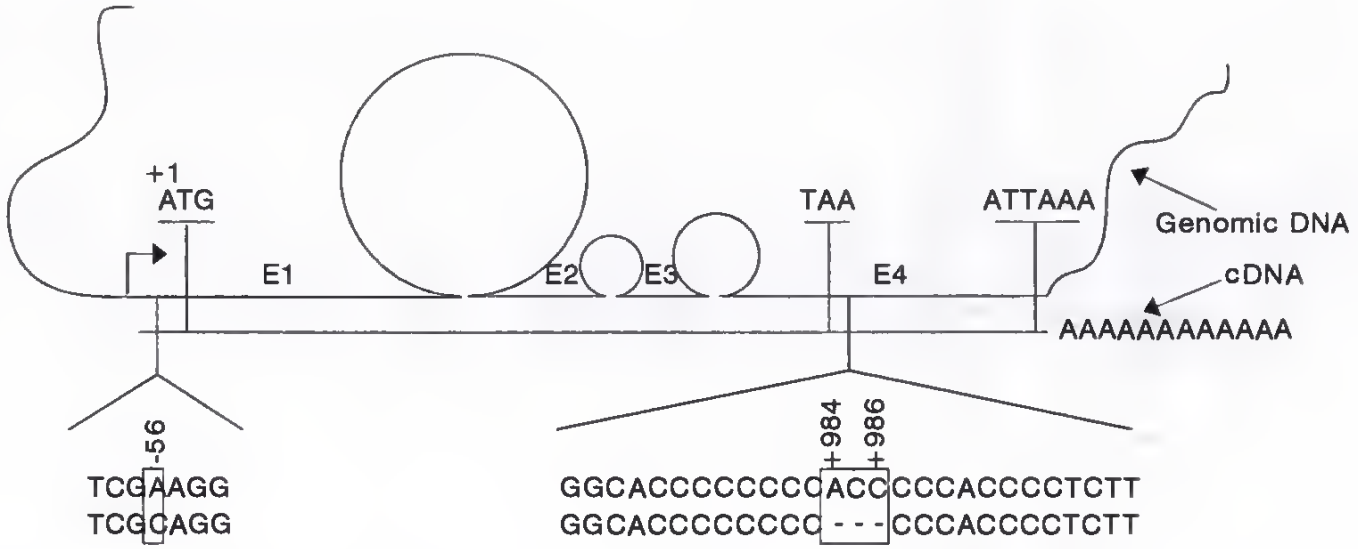


Figure 7-1. Schematic diagram of IGFBP-2 cDNA and genomic DNA. The positions are labeled relative to the translational initiation codon (+1). The parallel lines indicate identical sequences for cDNA and genomic exons. Note: lengths of exons are drawn to scale, but lengths of introns are not.

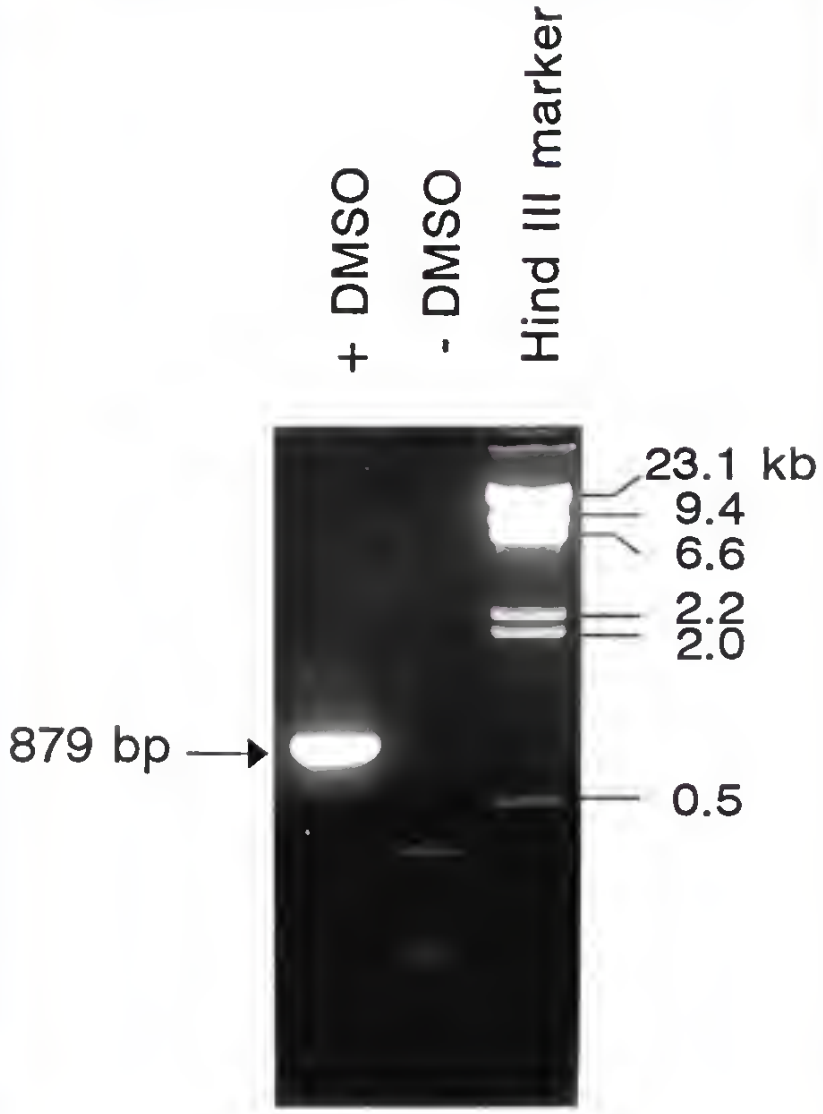


Figure 7-2. PCR amplification of the cDNA fragment encoding mature IGFBP-2. Left lane is PCR product obtained when DMSO was present in the PCR reaction. Middle lane is PCR product obtained when DMSO was omitted from the reaction. Right lane are the λ *Hind* III markers.

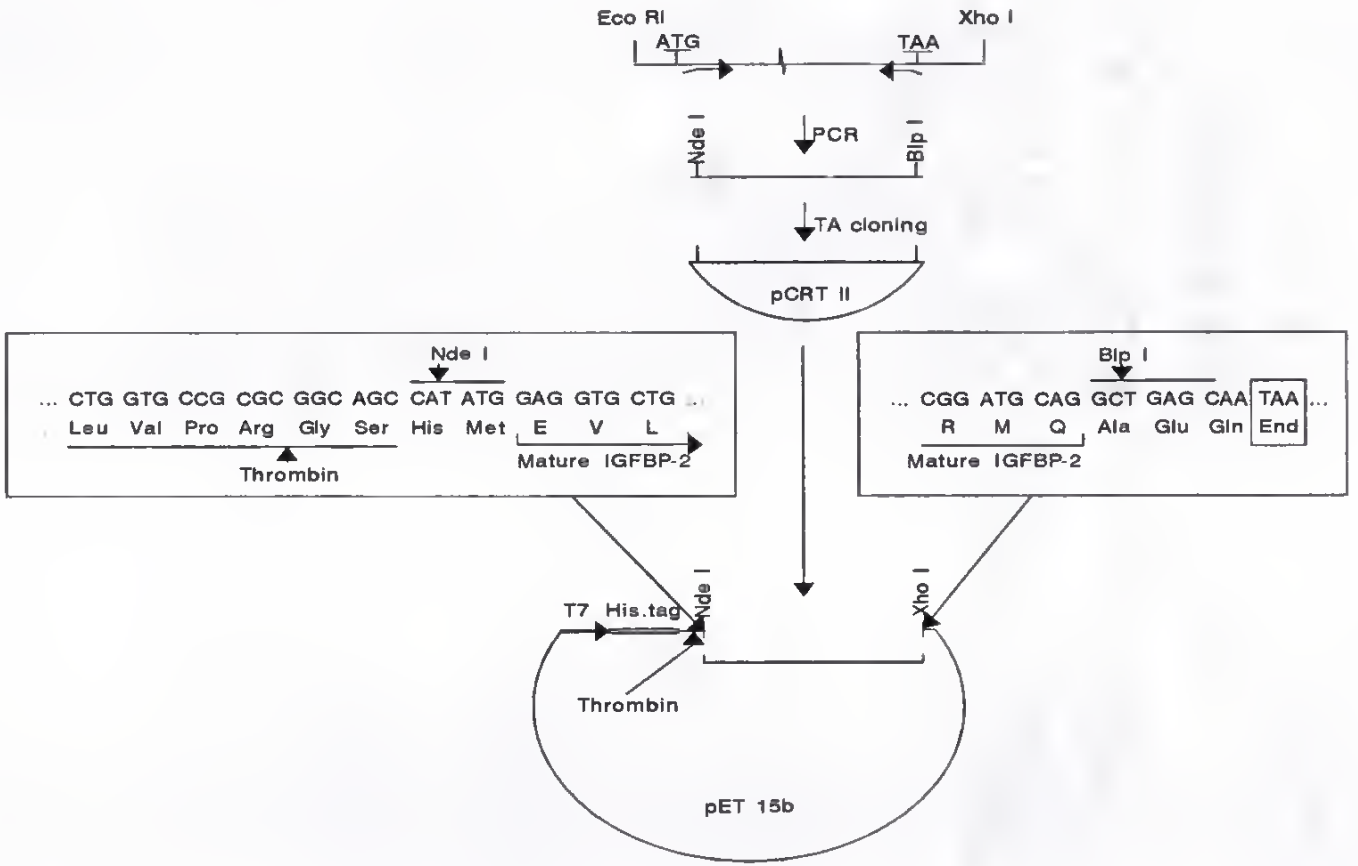


Figure 7-3. Scheme used for the generation of an expression construct encoding for recombinant porcine IGFBP-2.

Expression and Purification of Recombinant Porcine IGFBP-2

The plasmid DNA from clones BP2-48-I, BP2-49-I and BP2-50-I were separately used in transformation of BL21(DE3) cells (Novagen, Inc., Madison, WI). Three corresponding clones BP2exp-48, BP2exp-49 and BP2exp-50, were obtained and those were used for expression and subsequent purification of rpIGFBP-2 protein. After IPTG induction, the recombinant proteins from these cultures were purified as described in Materials and Methods. Samples obtained from different steps of the affinity column chromatography process were subjected to SDS-PAGE and Coomassie blue staining (Figure 7-4). As expected, the size of the recombinant mature IGFBP-2 fused to the 18 amino acid histidine tag was 34 kDa. This protein was highly enriched in the final eluate fraction. When samples from supernatant and the inclusion bodies were subjected to SDS-PAGE and Coomassie blue staining, most of the recombinant protein was in the supernatant indicating that the recombinant protein is soluble in the E.coli cells (Data not shown).

Functional Characterization of Recombinant IGFBP-2

Proteins from BP2exp-48, -49 and -50 cell extracts were subjected to ligand blot analysis to test for functional IGF binding of the recombinant proteins. The recombinant IGFBP-2 successfully bound IGF-I ligand (Figure 7-5), indicating that the IGFBP-2 had functional IGF-binding activity. Ligand blot analysis also indicated that expressed IGFBP-2 was mainly present in the soluble fraction (supernatant). Small amount of this protein was observed in the inclusion bodies.

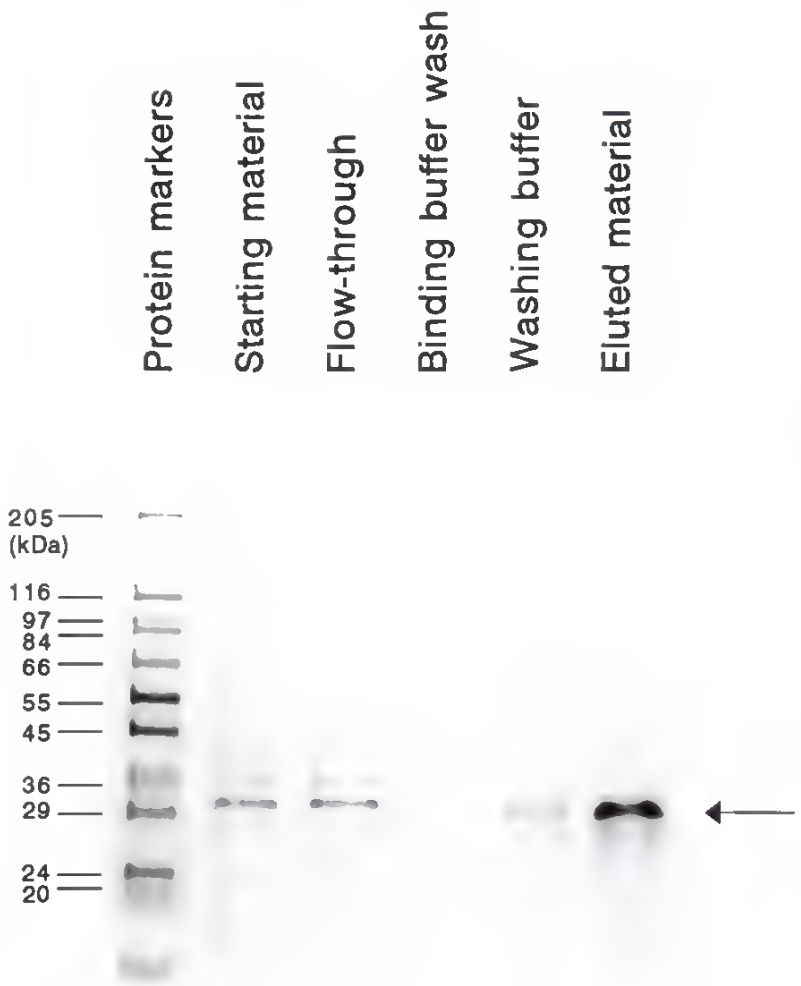


Figure 7-4. Enrichment of recombinant IGFBP-2. Lane 1 (from left), protein markers (marker); lane 2, proteins in the supernatant prior to loading on the affinity column (Before column); lane 3, proteins in the initial flow-through of the column (After column); lane 4, sample from wash-through with binding buffer; lane 5, sample from wash-through with wash buffer; lane 6, eluted sample. For each fraction an equivalent volume (30 μl) was electrophoresed in a 10% SDS-PAGE gel and stained with Coomassie blue.

Figure 7-5. Recombinant IGFBP-2 binds IGF-I. Samples from inclusion bodies (I) and soluble fractions (S) of sonicated BP2exp-48 (48), BP2exp-49 (49) and BP2exp-50 (50) cells were subjected to ligand blot analysis with ^{125}I-labeled IGF-I ligand.

Discussion

The cDNA coding sequence were amplified by polymerase chain reaction (PCR). Dimethylsuloxide (DMSO) was critical for this amplification. This optimized PCR condition may prove useful for other synthetic amplifications of this gene, such as reverse transcription PCR (RTPCR) for gene expression, and PCR for mutagenesis. To ensure the accuracy of amplification, three different cDNA clones were isolated and characterized by sequencing. The sequences from those three clones were identical to the genomic DNA sequences reported in Chapter 3.

In this study, full length porcine IGFBP-2 with an attached histidine tag was overexpressed in E. coli. The recombinant fusion protein consists of 278 amino acids of deduced mature IGFBP-2 sequence (Chapter 3), 24 additional amino acids at the N-terminus (including 17 residues of the histidine tag) and 3 additional amino acids at the C-terminus. The expected molecular mass for this fusion protein is 34,521 kDa. The size of this fusion protein estimated by SDS-PAGE is close to the predicted size of porcine IGFBP-2 and previously characterized native protein (Mondschein et al.,1990 and 1991; Colemanet al., 1991). Other evidence for identification of this protein is that its level is induced upon addition of isopropylthio-β-D-galactoside (IPTG) (data not shown).

In ligand blot analysis, the 34 kDa band strongly bound [^{125}I]IGF-I. This binding indicates that the 34 kDa band is the recombinant protein, and that this protein is biologically active, at least in terms of IGF binding. Although IGF-II binding of this protein has not been examined, it is anticipated that this protein will also bind to IGF-II since previous studies demonstrated that IGFBP-2 has higher affinity for IGF-II than for IGF-I (Bourner et al.,

1992). In the ligand blot analysis, extra bands of 70 kDa and various other sizes were also observed. The 70 kDa has been suggested to be a dimer in studies of human recombinant and mutated IGFBP-2 (Coulter et al., 1995). In the ligand blot analysis, proteins were subjected to nonreducing conditions. The intra-molecular disulfide bonds from 18 cysteines on each molecule could promote the aggregation of multiple molecules. Under physiological conditions, IGFBP-2 is secreted into body fluids, therefore intra-molecular interactions have less chance to occur and usually are not observed. However, in the overexpressed condition, larger amounts of these proteins accumulate in conditioned medium (Coulter et al., 1995) or cell cytosol (present study), facilitating intra-molecular interactions. This intra-molecular interaction may include formation of IGFBP-2 dimers or trimers, as well as complexes between IGFBP-2 and other proteins. The observation that the 70 kDa band is more prominent than others supports this speculative idea, since overexpressed IGFBP-2 has a much greater probability for dimerization than other interactions. The affinities of the dimer and the monomer for IGF binding cannot be estimated from the present study in the absence of known amounts of proteins formed.

As expected, most of the overexpressed rpIGFBP-2 was present in the supernatant of cell lysates . However, some of this protein was also observed in inclusion bodies. This phenomena was also observed in another E.coli expression study (Y. Wang, and R.C.M. Simmen, unpublished data). One possibility is that under the highly concentrated condition in the cells, aggregated proteins are preferentially sequestered within inclusion bodies.

Like IGFBP-1, IGFBP-2 contains a conserved RGD sequence in the C-terminal region (Drop., et al., 1992). This sequence commonly is seen on extracellular matrix (ECM)

proteins (Hass and Plow, 1994). This RGD sequence in IGFBP-1 interacts with the α5β1 integrin receptor on cell surfaces (Jones et al., 1993). Mutation of RGD to RGE in the sheep IGFBP-2 protein slightly affected the action of IGF-II (Delhanty and Han, 1993b). A more recent study showed that rat IGFBP-2 associates with cell membrane proteoglycans through its glycosaminoglycan binding domain (PKKLRP) (Russo et al., 1996), which is conserved in IGFBP-2 protein. Whether the RGD sequence of IGFBP-2 interacts with integrins, and the identity of such integrin(s), remain unknown. Data from Chapter 5 demonstrated that IGFBP-2 is associated with endometrial cell membranes. Availability of recombinant porcine IGFBP-2 will facilitate such functional studies.

Summary

In this study, an expression construct was generated that encodes the mature form of IGFBP-2 with small NH_2 and COOH extensions. The rpIGFBP-2 was expressed in and purified from E. coli, BL21(DE3) cells. The purified rpIGFBP-2 bound IGF-I.

CHAPTER 8
SUMMARY AND CONCLUSIONS

The present studies were focussed on clarifying the molecular basis for the developmental-, endometrial-specific, and hormonally-regulated IGFBP-2 gene transcription. As a first step, the chromosomal organization of the porcine IGFBP-2 gene was characterized in Chapter 3. Northern blot analysis confirmed that the endometrial IGFBP-2 gene exhibits relatively low expression during early pregnancy and maximal expression at mid-pregnancy. The transcript size was determined to be 1.6 kb. Using rat cDNA fragment as probe, a porcine genomic cosmid library was screened. Three positive cosmids 6-1, 4-9 and 9-7 were mapped. Cosmid 6-1 and 4-9 overlapped and encompassed the 5'-upstream region and exon 1 through to exon 3. Cosmid 9-7 covers exon 4 and 3'-downstream region. Subclones H6, H1-12, B11 and S29, from the above three cosmids, were subjected to sequence analysis. The intron 3 size was estimated by genomic PCR using primers corresponding to exon 3 and exon 4 sequences. The porcine IGFBP-2 gene spans ~29 kb, and is comprised of four exons and three introns. Exons 1, 2, 3 and 4 are ~526, 227, 141 and 528 bp, respectively. Introns 1, 2, 3 and 4 are ~24 kb, 746 bp and 2.6 kb, respectively. This gene encodes a 316 amino acid precursor with 18 consensus cysteines and an RGD sequence. An upstream sequence of 1.4 kb was obtained. This gene promoter lacks TATA and CAAT box motifs and contain two clusters of transcriptional start sites located at -109 and -87 nt relative to the first ATG

codon for the protein. This promoter contains consensus GC boxes as well as other upstream consensus region. Results from Chapter 3 provided a foundation for subsequent studies described in this research.

In Chapter 4, studies were focused on the identification of potential cis-regulatory regions and/or elements within the 1.4 kb 5'-upstream region. To accomplish this, four luciferase reporter gene constructs, containing different lengths of 5'-flanking sequences, were generated (Figure 4-1). These four constructs were transfected into human cancer cell lines (ECC-1 and JEG-3), and pregnant pig endometrial epithelial and stromal cells (D18GE, D30GE, D18ST and D30ST). Results of transient transfection indicated that: 1). A 110 bp region from -874 to -765 has transcriptional stimulatory activity, which is independent on cell-type. 2). A 523 bp region from -1397 to -875 and a 459 bp region from -764 to -306 appear to have transcriptional repressing activities, which are dependent on cell-type. 3). The region from -305 to +73 spans the basal promoter. To confirm the stimulatory activity of the 110 bp region, a construct [SaB(110)-pLUC], which contains this 110 bp fragment fused upstream of the SV40 promoter, was generated and transfected into D18GE cells. Results from this experiment showed that this 110 bp region increased the SV40 promoter activity by 5-fold (Figure 4-3).To identify the *cis*-acting element(s) within the 110 bp activating region, a series of gel retardation assays were performed. Two consensus sequences TCAGGG and CCCTGA, which bound the same nuclear protein designated A2 DNA binding protein, were identified. The A2 DNA binding protein has a molecular weight of 33 kDa and is present in endometria of pigs at all pregnancy stages as well as in other tissues.

In Chapter 5, steroid and peptide hormonal regulation of IGFBP-2 gene transcription was examined. Results from RNase protection assays showed: 1). Estrogen stimulated IGFBP-2 gene expression in the *in vivo*- ovariectomized pig model and *in vitro* in D18GE cells. 2). IGF-II, PMA and cAMP increased IGFBP-2 mRNA abundance in primary cultures of D18GE cells in a dose-dependent manner, while IGF-I and TGF-β1 had no effect. Ligand blot analysis showed that the binding capacity of IGF-II receptors on endometrial cells correlated with maximal gene expression of IGFBP-2. Evidence implicated fetal IGF-II is in the pregnancy-induced and pregnancy stage-specific expression of endometrial IGFBP-2, while estrogen is likely responsible for estrous cycle changes in IGFBP-2 expression. Estrogen and IGF-II response regions for these stimulatory effects could not be identified within the 1.4 kb upstream region. In contrast, an inhibitory region, which responded to estrogen, progesterone and IGF-II treatments, was identified in the region from 764 to +73 relative to the ATG. Based on these observations, a speculative model for IGFBP-2 gene regulation was presented (Figure 5-11).

The objective of the studies described in Chapter 6 was to clarify the possibility that DNA methylation of the GC rich promoter region may affect tissue-specific regulation of IGFBP-2 gene . Computer-assisted sequence analysis revealed that all of the IGFBP-2 exonic sequences are GC rich, and that exon 1 and flanking region are embedded in a CpG island. Genomic Southern blot with DNA from myometrium, endometrium and placenta from Day 30 pregnant pigs, showed no differences in DNA methylation patterns between high and low IGFBP-2 expressing tissues. *In vitro* methylation of the promoter region (-305 to +73) did not alter the transcriptional activity when examined in an *in vitro* transcription system.

Results from these studies indicated that DNA methylation may not constitute a major factor in IGFBP-2 gene regulation.

Lastly, biologically active recombinant porcine IGFBP-2 (rpIGFBP-2) was expressed in and purified from E. coli (Chapter 7). The expression vector was generated after PCR amplification of cDNA encoding the mature protein. Ligand blot analysis demonstrated that the rpIGFBP-2 bound IGF-I. This rpIGFBP-2 will be useful for further functional studies of IGFBP-2.

In conclusion: 1). Distal regions of the IGFBP-2 gene promoter can mediate IGFBP-2 gene transcription. A 110 bp region from -874 to 765 has transcriptional stimulatory activity, in which are present two consensus elements TCAGGG and CCCTGA that can bind a 33 kDa nuclear protein, designated herein as, A2 DNA binding protein. 2). IGF-II may be responsible for pregnancy-induced and pregnancy stage-specific IGFBP-2 gene expression, while estrogen may be responsible for estrous cycle-dependent changes in IGFBP-2 gene expression. 3). PKA and PKC signal transduction pathways are involved in activation of IGFBP-2 transcription. 4). Although the IGFBP-2 gene is GC rich, DNA methylation may not constitute a major factor underlying transcriptional regulation of this gene *in utero*. 5). Bacterially-expressed rpIGFBP-2 bound IGF-I and constitutes an important reagent for studying uterine IGFBP-2 functions in pregnancy.

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BIOGRAPHICAL SKETCH

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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